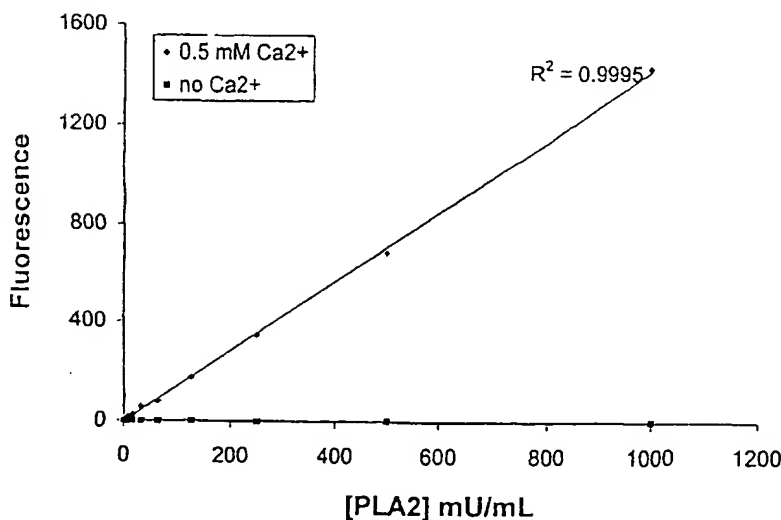




## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>7</sup> : C09B 11/24, G01N 33/533, 33/58, 33/542, C12Q 1/68, C07H 21/00 // C09B 11/02, C12Q 1/34, 1/37		A1	(11) International Publication Number: WO 00/64988 (43) International Publication Date: 2 November 2000 (02.11.00)
(21) International Application Number: PCT/US00/10740 (22) International Filing Date: 21 April 2000 (21.04.00) (30) Priority Data: 60/130,808 23 April 1999 (23.04.99) US (71) Applicant: MOLECULAR PROBES, INC. [US/US]; 4849 Pitchford Avenue, Eugene, OR 97402-9165 (US). (72) Inventors: HAUGLAND, Richard, P.; 2233 Lasater, Eugene, OR 97405 (US). SINGER, Victoria, L.; 910 East 23rd Avenue, Eugene, OR 97405 (US). YUE, Stephen, T.; 2940 West 19th Avenue, Eugene, OR 97405 (US). (74) Agents: HELFENSTEIN, Allegra, J. et al.; Molecular Probes, Inc., 4849 Pitchford Avenue, Eugene, OR 97402 (US).		(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.	

(54) Title: XANTHENE DYES AND THEIR APPLICATION AS LUMINESCENCE QUENCHING COMPOUNDS



## 57) Abstract

The quenching compounds of the invention are nitrogen-substituted xanthenes that are substituted by one or more aromatic or heteroaromatic quenching moieties. The quenching compounds of the invention exhibit little or no observable fluorescence and efficiently quench a broad spectrum of luminescent compounds. The chemically reactive quenching compounds possess utility for labeling a wide variety of substances, including biomolecules. These labeled substances are highly useful for a variety of energy-transfer assays and applications.

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## XANTHENE DYES AND THEIR APPLICATION AS LUMINESCENCE QUENCHING COMPOUNDS

### FIELD OF THE INVENTION

5 The invention relates to xanthene compounds that are efficient quenchers of luminescence, and their precursors. Chemically reactive versions of the xanthene compounds are described, as are conjugates prepared using the reactive compounds. Applications using the xanthene quenching compounds are also described.

### BACKGROUND

Fluorescence Resonance Energy Transfer (FRET) is a process whereby a first fluorescent dye (the "donor" dye) is excited, typically by illumination, and transfers its absorbed energy to a second dye (the "acceptor" dye) that has a longer wavelength and therefore lower energy emission. Where the second dye is fluorescent, energy transfer results in fluorescence emission at the wavelength of the second dye. However, where the second dye is nonfluorescent, the absorbed energy does not result in fluorescence emission, and the fluorescence of the initial donor dye is said to be "quenched". Energy transfer can also be utilized to quench the emission of luminescent donors, including phosphorescent and chemiluminescent donors. When a luminescent emission is restored by preventing energy transfer, the luminescence is said to be "dequenched" or "unquenched".

FRET has been utilized to study DNA hybridization and amplification, the dynamics of protein folding, proteolytic degradation, and interactions between other biomolecules. By far the most common donor-acceptor dye pair utilized for these applications is dabcyI (the quenching dye) and EDANS (the fluorophore) (as discussed in The Molecular Probes Handbook of Fluorescence Probes and Research Chemicals, 1996, chapter 9.3).

Despite the widespread use of the dabcyI-EDANS energy transfer pair, this technology possesses a number of shortcomings, including cellular autofluorescence, DNA cross-linking, and strong intrinsic absorptions of many drugs and biologically active proteins. Both dabcyI and EDANS have low extinction coefficients, resulting in assays that are comparatively insensitive.

In order to avoid the difficulties associated with the use of ultraviolet excitation, the absorption of the energy acceptor should be closely aligned with the visible light fluorophore used. The compounds of the instant invention have been discovered to quench the fluorescence of a large variety of dyes, including dyes that are excited in the ultraviolet, but also including fluoresceins, rhodamines, and even longer wavelength fluorophores such as CY 5 and allophycocyanin. In addition, the compounds of the invention have significantly larger extinction coefficients than the quenching compounds typically used in energy transfer assays.

The compounds of the instant invention represent a new and highly useful class nonfluorescent energy acceptors, including chemically reactive versions, and the conjugates prepared therefrom.

## 5 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Correlation of fluorescence intensity with phospholipase activity of PLA<sub>2</sub> (as described in Example 40).

## 10 SUMMARY OF THE INVENTION AND DESCRIPTION OF PREFERRED EMBODIMENTS

The compounds of the invention are derivatives of 3- and/or 6-amino xanthenes that are substituted at one or more amino nitrogen atoms by an aromatic or heteroaromatic quenching moiety, Q. The quenching compounds of the invention have absorption maxima above 530 nm, have  
15 little or no observable fluorescence and efficiently quench a broad spectrum of luminescence, such as is emitted by chemilumiphores, phosphors, or fluorophores. In one embodiment, the quenching compound is a substituted rhodamine. In another embodiment, the quenching compound is a substituted rhodol. The chemically reactive quenching compounds possess utility for labeling a wide variety of substances, including biomolecules. These labeled substances are highly useful for a  
20 variety of energy-transfer assays and applications, particularly when used in combination with a luminophore.

As used herein, each quenching moiety, Q, is an aromatic or heteroaromatic ring system, having 1-4 fused aromatic or heteroaromatic rings, attached to the amino nitrogen by a single covalent bond. Where the Q moiety is fully aromatic and contains no heteroatom, Q comprises 1-4  
25 fused six-membered aromatic rings. Where the Q moiety is heteroaromatic, Q incorporates at least one 5- or 6-membered aromatic heterocycle that contains at least 1 and as many as 4 heteroatoms that are selected from the group consisting of O, N, and S in any combination, that is optionally fused to an additional six-membered aromatic ring, or is fused to one 5- or 6-membered heteroaromatic ring that contains at least 1 and as many as 3 heteroatoms that are selected from the  
30 group consisting of O, N, and S in any combination.

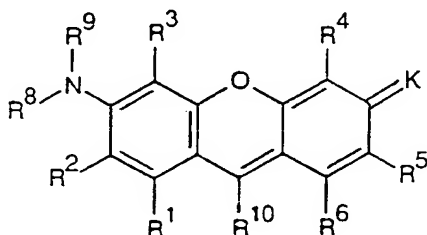
Each Q moiety is bound to the xanthene compounds of the invention at a 3- or 6-amino nitrogen atom via a single covalent bond. In some embodiments, the amino nitrogen substituents, taken in combination, form a 5- or 6-membered heterocycle that is a piperidine, a morpholine, a pyrrolidine, a pyrazine, or a piperazine, and the Q moiety is fused to the resulting heterocycle  
35 adjacent to the xanthene nitrogen, so as to be formally bound to the amino nitrogen via single bond. The Q moiety may be bound to the amino nitrogen atom at either an aromatic or heteroaromatic ring, provided it is attached at a carbon atom of that ring.

Typically, the Q moieties of the invention are substituted or unsubstituted phenyl, naphthyl,

anthracenyl, benzothiazole, benzoxazole, or benzimidazole. Where the amino nitrogen substituents form a 5- or 6-membered heterocycle and the Q moiety is fused to the resulting heterocycle, the heterocycle is typically a pyrrolidine ring and the Q moiety is typically a fused six-membered aromatic ring. Most preferably, Q is a phenyl or substituted phenyl.

- 5 Each Q moiety is optionally and independently substituted by hydrogen, halogen, cyano, sulfo, alkali or ammonium salt of sulfo, carboxy, alkali or ammonium salt of carboxy, nitro, alkyl, perfluoroalkyl, alkoxy, alkylthio, amino, monoalkylamino, dialkylamino or alkylamido.

The quenching compounds of the invention have the formula



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wherein the K moiety is O or N-R<sup>16</sup>R<sup>17</sup>.

- For all the compounds of the invention, at least one of R<sup>8</sup>, R<sup>9</sup>, R<sup>18</sup> and R<sup>19</sup> is a Q moiety. Alternatively, either R<sup>8</sup> taken in combination with R<sup>9</sup>, or R<sup>18</sup> taken in combination with R<sup>19</sup>, forms a saturated 5- or 6-membered heterocycle that is a piperidine, or a pyrrolidine that is fused to a Q moiety. Typically one of R<sup>8</sup> and R<sup>9</sup> and one of R<sup>18</sup> and R<sup>19</sup> are each a Q moiety, which are the same or different. In another embodiment, each of R<sup>8</sup>, R<sup>9</sup>, R<sup>18</sup> and R<sup>19</sup> is a Q moiety, which may be the same or different.

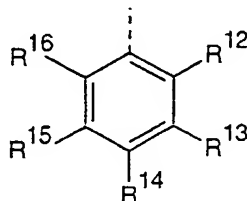
- The remainder of R<sup>8</sup>, R<sup>9</sup>, R<sup>18</sup>, and R<sup>19</sup> are independently H, C<sub>1</sub>-C<sub>6</sub> alkyl, C<sub>1</sub>-C<sub>6</sub> carboxyalkyl, C<sub>1</sub>-C<sub>6</sub> sulfoalkyl, a salt of C<sub>1</sub>-C<sub>6</sub> carboxyalkyl, or a salt of C<sub>1</sub>-C<sub>6</sub> sulfoalkyl, wherein the alkyl portions are optionally substituted by amino, hydroxy, carboxylic acid, a salt of carboxylic acid, or a carboxylic acid ester of a C<sub>1</sub>-C<sub>6</sub> alkyl. Alternatively, where R<sup>8</sup> in combination with R<sup>9</sup>, or R<sup>18</sup> in combination with R<sup>19</sup>, or both, forms a saturated 5- or 6-membered heterocyclic ring that is a piperidine, a morpholine, a pyrrolidine, a pyrazine, or a piperazine, that is optionally substituted by methyl, sulfonic acid, a salt of sulfonic acid, carboxylic acid, a salt of carboxylic acid, or a carboxylic acid ester of a C<sub>1</sub>-C<sub>6</sub> alkyl. Alternatively, one or more of R<sup>8</sup> in combination with R<sup>9</sup>, R<sup>9</sup> in combination with R<sup>18</sup>, R<sup>18</sup> in combination with R<sup>4</sup>, or R<sup>19</sup> in combination with R<sup>5</sup>, forms a 5- or 6-membered ring that is saturated or unsaturated, and that is optionally substituted by one or more C<sub>1</sub>-C<sub>6</sub> alkyls or -CH<sub>2</sub>SO<sub>3</sub>X, where X is H or a counterion.

- R<sup>1</sup> and R<sup>5</sup> are H, or one or more of R<sup>1</sup> in combination with R<sup>2</sup>, or R<sup>5</sup> in combination with R<sup>5</sup>, is a fused six-membered aromatic ring.

Substituents R<sup>2</sup>, R<sup>3</sup>, R<sup>4</sup>, and R<sup>5</sup> are independently H, F, Cl, Br, I, CN; or C<sub>1</sub>-C<sub>18</sub> alkyl, or C<sub>1</sub>-

C<sub>18</sub> alkoxy, where each alkyl or alkoxy is optionally further substituted by F, Cl, Br, I, a carboxylic acid, a salt of carboxylic acid, or a carboxylic acid ester of a C<sub>1</sub>-C<sub>6</sub> alcohol; or -SO<sub>3</sub>X.

The pendant group R<sup>10</sup> is H, CN, a carboxylic acid, a salt of carboxylic acid, or a carboxylic acid ester of a C<sub>1</sub>-C<sub>6</sub> alcohol. Alternatively R<sup>10</sup> is a saturated or unsaturated, branched or  
 5 unbranched C<sub>1</sub>-C<sub>18</sub> alkyl that is optionally substituted one or more times by F, Cl, Br, carboxylic acid, a salt of carboxylic acid, a carboxylic acid ester of a C<sub>1</sub>-C<sub>6</sub> alcohol, -SO<sub>3</sub>X, amino, alkylamino, or dialkylamino, the alkyl groups of which have 1-6 carbons. In another preferred embodiment, R<sup>10</sup> has the formula

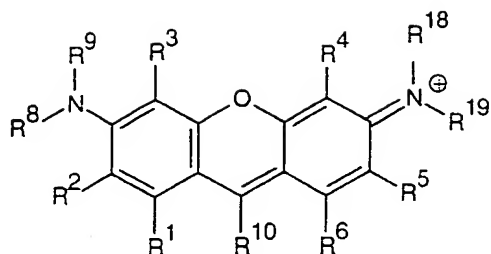


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where R<sup>12</sup>, R<sup>13</sup>, R<sup>14</sup>, R<sup>15</sup> and R<sup>16</sup> are independently H, F, Cl, Br, I, -SO<sub>3</sub>X, a carboxylic acid, a salt of carboxylic acid, CN, hydroxy, amino, hydrazino, azido; or C<sub>1</sub>-C<sub>18</sub> alkyl, C<sub>1</sub>-C<sub>18</sub> alkoxy, C<sub>1</sub>-C<sub>18</sub> alkylthio, C<sub>1</sub>-C<sub>18</sub> alkanoylamino, C<sub>1</sub>-C<sub>18</sub> alkylaminocarbonyl, C<sub>2</sub>-C<sub>36</sub> dialkylaminocarbonyl, C<sub>1</sub>-C<sub>18</sub>  
 15 alkylloxycarbonyl, or C<sub>7</sub>-C<sub>18</sub> arylcarboxamido, the alkyl or aryl portions of which are optionally substituted one or more times by F, Cl, Br, I, hydroxy, carboxylic acid, a salt of carboxylic acid, a carboxylic acid ester of a C<sub>1</sub>-C<sub>6</sub> alcohol, -SO<sub>3</sub>X, amino, alkylamino, dialkylamino or alkoxy, the alkyl portions of each having 1-6 carbons. Alternatively, a pair of adjacent substituents R<sup>13</sup> and R<sup>14</sup>, R<sup>14</sup> and R<sup>15</sup>, or R<sup>15</sup> and R<sup>16</sup>, taken in combination, form a fused 6-membered aromatic ring that is  
 20 optionally further substituted by carboxylic acid, or a salt of carboxylic acid.

The compounds of the invention are optionally substituted by a reactive group (R<sub>x</sub>) or conjugated substance (S<sub>c</sub>) that is attached to the compound of the invention by a covalent linkage, L. Typically, the compound of the invention is substituted by an -L-R<sub>x</sub> or -L-S<sub>c</sub> moiety at one or more of R<sup>8</sup>, R<sup>9</sup>, R<sup>12</sup>, R<sup>13</sup>, R<sup>14</sup>, R<sup>15</sup>, R<sup>16</sup>, R<sup>18</sup>, or R<sup>19</sup>, preferably at one of R<sup>12</sup>-R<sup>16</sup>, most preferably at R<sup>12</sup>, R<sup>14</sup> or  
 25 R<sup>15</sup>, or as a substituent on a Q moiety. Alternatively, an -L-R<sub>x</sub> or -L-S<sub>c</sub> moiety is present as a substituent on an alkyl, alkoxy, alkylthio or alkylamino substituent. In one embodiment, exactly one of R<sup>8</sup>, R<sup>9</sup>, R<sup>12</sup>, R<sup>13</sup>, R<sup>14</sup>, R<sup>15</sup>, R<sup>16</sup>, R<sup>18</sup>, or R<sup>19</sup> is an -L-R<sub>x</sub> or -L-S<sub>c</sub> moiety. In another embodiment, exactly one of R<sup>12</sup>, R<sup>13</sup>, R<sup>14</sup>, R<sup>15</sup>, or R<sup>16</sup> is an -L-R<sub>x</sub> or -L-S<sub>c</sub> moiety. In a preferred embodiment, one of R<sup>12</sup>, R<sup>14</sup>, and R<sup>15</sup> is an -L-R<sub>x</sub> or an -L-S<sub>c</sub> moiety.

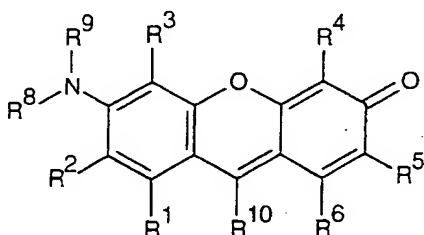
30 Where the K moiety is N<sup>+</sup>R<sup>18</sup>R<sup>19</sup>, the compounds of the invention are rhodamines, and have the formula



wherein at least one of  $R^8$ ,  $R^9$ ,  $R^{18}$  and  $R^{19}$  is a Q moiety. Preferably at least one of  $R^8$  and  $R^9$  is a Q moiety and at least one of  $R^{18}$  and  $R^{19}$  is a Q moiety, which may be the same or different.

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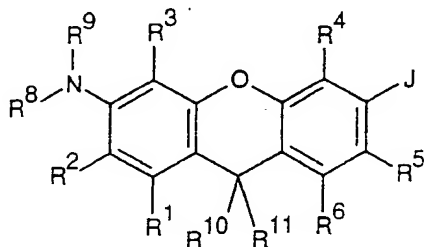
Where the K moiety is O, the compounds of the invention are rhodols, and have the formula



wherein at least one of  $R^8$  and  $R^9$  is a Q moiety.

Generally colorless forms of the instant compounds have the formula

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wherein J is  $O-R^7$  or  $NR^{18}R^{19}$ , and  $R^1 - R^{19}$  is as defined above.

These precursors to the quenching compounds of the invention typically do not function as quenchers unless or until the aromaticity of the ring system is restored, as for the quenching compounds described above. In these precursors  $R^7$  is H,  $C_1-C_6$  alkyl,  $C_1-C_6$  carboxyalkyl,  $C_1-C_6$  sulfoalkyl, a salt of  $C_1-C_6$  carboxyalkyl, or a salt of  $C_1-C_6$  sulfoalkyl, wherein the alkyl portions are optionally substituted by amino, hydroxy, carboxylic acid, a salt of carboxylic acid, or a carboxylic acid ester of a  $C_1-C_6$  alkyl. Alternatively,  $R^7$  is a monovalent radical formally derived by removing a hydroxy group from a carboxylic acid, a sulfonic acid, a phosphoric acid, or a mono- or polysaccharide, such as a glycoside. In another embodiment,  $R^7$  is a photolabile caging group, such

as described in Haugland, MOLECULAR PROBES HANDBOOK OF FLUORESCENT PROBES AND RESEARCH CHEMICALS, Sixth Ed., Chapter 19 (1996).

R<sup>10</sup> is as defined previously, and R<sup>11</sup> is H, hydroxy, CN or alkoxy having 1-6 carbons. Alternatively, R<sup>10</sup> in combination with R<sup>11</sup> forms a 5- or 6-membered spirolactone ring, or R<sup>11</sup> in combination with R<sup>12</sup> forms a 5- or 6-membered spirolactone ring, or a 5- or 6-membered sultone ring.

These precursor compounds are readily converted to the fully conjugated quenching compounds of the invention by chemical, enzymatic, or photolytic means. Typically, the colorless precursors are substituted by an -L-R<sub>x</sub> moiety, or are conjugated to a desired substance (Sc).

#### Conjugates of Reactive Compounds

In a preferred embodiment of the invention, the compound of the invention (quenching compound or precursor compound) is substituted by at least one group -L-R<sub>x</sub>, where R<sub>x</sub> is the reactive group that is attached to the compound of the invention by a covalent linkage L. The compounds of the invention with a reactive group (R<sub>x</sub>) label a wide variety of organic or inorganic substances that contain or are modified to contain functional groups with suitable reactivity, resulting in chemical attachment of the conjugated substance (Sc), represented by -L-Sc.

The reactive group and functional group are typically an electrophile and a nucleophile that can generate a covalent linkage. Typically, the conjugation reaction between the reactive compound and the substance to be conjugated results in one or more atoms of the reactive group R<sub>x</sub> to be incorporated into a new linkage L attaching the compound to the conjugated substance Sc. Selected examples of functional groups and linkages are shown in Table 2, where the reaction of an electrophilic group and a nucleophilic group yields a covalent linkage.

Table 2: Examples of some routes to useful covalent linkages

Electrophilic Group	Nucleophilic Group	Resulting Covalent Linkage
activated esters*	amines/anilines	carboxamides
acrylamides	thiols	thioethers
acyl azides**	amines/anilines	carboxamides
acyl halides	amines/anilines	carboxamides
acyl halides	alcohols/phenols	esters
acyl nitriles	alcohols/phenols	esters
acyl nitriles	amines/anilines	carboxamides
aldehydes	amines/anilines	imines
aldehydes or ketones	hydrazines	hydrazones



aldehydes or ketones	hydroxylamines	oximes
alkyl halides	amines/anilines	alkyl amines
alkyl halides	carboxylic acids	esters
alkyl halides	thiols	thioethers
alkyl halides	alcohols/phenols	ethers
alkyl sulfonates	thiols	thioethers
alkyl sulfonates	carboxylic acids	esters
alkyl sulfonates	alcohols/phenols	ethers
anhydrides	alcohols/phenols	esters
anhydrides	amines/anilines	carboxamides
aryl halides	thiols	thiophenols
aryl halides	amines	aryl amines
aziridines	thiols	thioethers
boronates	glycols	boronate esters
carboxylic acids	amines/anilines	carboxamides
carboxylic acids	alcohols	esters
carboxylic acids	hydrazines	hydrazides
carbodiimides	carboxylic acids	N-acylureas or anhydrides
dialkyl azoalkanes	carboxylic acids	esters
epoxides	thiols	thioethers
haloacetamides	thiols	thioethers
haloplatinate	amino	platinum complex
haloplatinate	heterocycle	platinum complex
halotriazines	amines/anilines	aminotriazines
halotriazines	alcohols/phenols	triazinyl ethers
imido esters	amines/anilines	amidines
isocyanates	amines/anilines	ureas
isocyanates	alcohols/phenols	urethanes
isothiocyanates	amines/anilines	thioureas
maleimides	thiols	thioethers
phosphoramidites	alcohols	phosphite esters
silyl halides	alcohols	silyl ethers
sulfonate esters	amines/anilines	alkyl amines
sulfonate esters	thiols	thioethers
sulfonate esters	carboxylic acids	esters
sulfonate esters	alcohols	ethers

sulfonyl halides	amines/anilines	sulfonamides
sulfonyl halides	phenols/alcohols	sulfonate esters

\* Activated esters, as understood in the art, generally have the formula -COW, where W is a good leaving group (e.g. succinimidyl (-OC<sub>4</sub>H<sub>4</sub>O<sub>2</sub>) sulfosuccinimidyl (-OC<sub>4</sub>H<sub>3</sub>O<sub>2</sub>-SO<sub>3</sub>H), -1-oxybenzotriazolyl (-OC<sub>6</sub>H<sub>4</sub>N<sub>3</sub>); 4-sulfo-2,3,5,6-tetrafluorophenyl; or an aryloxy group or aryloxy substituted one or more times by electron withdrawing substituents such as nitro, fluoro, chloro, cyano, or trifluoromethyl, or combinations thereof, used to form activated aryl esters; or a carboxylic acid activated by a carbodiimide to form an anhydride or mixed anhydride -OCOR<sup>a</sup> or -OCNR<sup>a</sup>NHR<sup>b</sup>, where R<sup>a</sup> and R<sup>b</sup>, which may be the same or different, are C<sub>1</sub>-C<sub>6</sub> alkyl, C<sub>1</sub>-C<sub>6</sub> perfluoroalkyl, or C<sub>1</sub>-C<sub>6</sub> alkoxy; or cyclohexyl, 3-dimethylaminopropyl, or N-morpholinoethyl).

\*\* Acyl azides can also rearrange to isocyanates

The covalent linkage L binds the reactive group R<sub>x</sub> or conjugated substance S<sub>c</sub> to the compound, either directly (L is a single bond) or with a combination of stable chemical bonds, optionally including single, double, triple or aromatic carbon-carbon bonds, as well as carbon-nitrogen bonds, nitrogen-nitrogen bonds, carbon-oxygen bonds, carbon-sulfur bonds, phosphorus-oxygen bonds, and phosphorus-nitrogen bonds. L typically includes ether, thioether, carboxamide, sulfonamide, urea, urethane or hydrazine moieties. In one embodiment, the covalent linkage incorporates a platinum atom, such as described in U.S. Patent No. 5,714,327. Preferred L moieties have 1-20 nonhydrogen atoms selected from the group consisting of C, N, O, P, and S; and are composed of any combination of ether, thioether, amine, ester, carboxamide, sulfonamide, hydrazide bonds and aromatic or heteroaromatic bonds. Preferably L is a combination of single carbon-carbon bonds and carboxamide or thioether bonds. The longest linear segment of the linkage L preferably contains 4-10 nonhydrogen atoms, including one or two heteroatoms. Examples of L include substituted or unsubstituted polymethylene, arylene, alkylarylene, arylenealkyl, or arylthio. In one embodiment, L contains 1-6 carbon atoms; in another, L is a thioether linkage. In yet another embodiment, L is or incorporates the formula -(CH<sub>2</sub>)<sub>a</sub>(CONH(CH<sub>2</sub>)<sub>b</sub>)<sub>z</sub>, where a has any value from 0-5, b has any value from 1-5 and z is 0 or 1.

Typically, R<sub>x</sub> will react with an amine, a thiol, an alcohol, an aldehyde or a ketone. In one embodiment, R<sub>x</sub> is an acrylamide, an activated ester of a carboxylic acid, an acyl azide, an acyl nitrile, an aldehyde, an alkyl halide, an amine, an anhydride, an aniline, an aryl halide, an azide, an aziridine, a boronate, a carboxylic acid, a diazoalkane, a haloacetamide, a halotriazine, a hydrazine (including hydrazides), an imido ester, an isocyanate, an isothiocyanate, a maleimide, a phosphoramidite, a reactive platinum complex, a sulfonyl halide, or a thiol group. By "reactive platinum complex" is meant chemically reactive platinum complexes such as described in U.S. Patent No. 5,714,327.

Preferably, R<sub>x</sub> is a phosphoramidite, a succinimidyl ester of a carboxylic acid, a haloacetamide, a hydrazine, an isothiocyanate, a maleimide group, a perfluorobenzamido, or an azidoperfluorobenzamido group. More preferably, R<sub>x</sub> is a phosphoramidite, a reactive platinum complex, or a succinimidyl ester of a carboxylic acid. Where R<sub>x</sub> is a reactive platinum complex, it is typically a haloplatinate.

Where the reactive group is a photoactivatable group, such as an azide, diazirinyl, azidoaryl, azidoperfluoroaryl, or a psoralen derivative, the compound of the invention typically becomes chemically reactive only after illumination with light of an appropriate wavelength.

Where Rx is an activated ester of a carboxylic acid (such as a succinimidyl ester), the reactive compound of the invention is particularly useful for preparing conjugates of proteins, polysaccharides, lipids, nucleotides, or amino-modified oligonucleotides or haptens. Where Rx is a maleimide or haloacetamide, the reactive compound of the invention is particularly useful for conjugation to thiol-containing substances. Where Rx is a hydrazide, the reactive compound of the invention is particularly useful for conjugation to periodate-oxidized carbohydrates and glycoproteins. Where Rx is a phosphoramidite, the reactive compound of the invention is particularly useful for the preparation of conjugates of oligonucleotides.

The reactive compounds of the invention are useful for the preparation of any conjugated substance that possess a suitable functional group for covalent attachment of the compound. Examples of particularly useful conjugates include, among others, conjugates of antigens, steroids, vitamins, drugs, haptens, metabolites, toxins, environmental pollutants, amino acids, peptides, proteins, nucleotides, oligonucleotides, nucleic acids (nucleic acid polymers), carbohydrates, lipids, ion-complexing moieties, and non-biological polymers. Preferably the conjugated substance is an amino acid, peptide, protein, nucleotide, oligonucleotide, or nucleic acid polymer. In particular, the conjugated substance is preferably a hapten or a member of a specific binding pair.

Choice of the reactive group used to attach the compound of the invention to the substance to be conjugated typically depends on the functional group on the substance to be conjugated and the type or length of covalent linkage desired. The types of functional groups typically present on the organic or inorganic substances include, but are not limited to, amines, thiols, alcohols, phenols, aldehydes, ketones, phosphates, imidazoles, hydrazines, hydroxylamines, disubstituted amines, halides, epoxides, sulfonate esters, purines, pyrimidines, carboxylic acids, or a combination of these groups. A single type of reactive site may be available on the substance (typical for polysaccharides), or a variety of sites may occur (e.g. amines, thiols, alcohols, phenols), as is typical for proteins. A given conjugated substance may be conjugated to one or more compound of the invention, which may be the same or different, or to a substance that is additionally modified by a hapten or specific binding pair member. Although some selectivity in cross-reactivity can be obtained by careful control of the reaction conditions, selectivity of labeling is best obtained by selection of an appropriate reactive functional group.

In a preferred embodiment, the conjugated substance is additionally conjugated to one or more luminophores, which may be the same or different. In one embodiment, energy transfer from the luminophores to the quenching compound occurs, resulting in significant quenching of luminescence. In another embodiment, the luminophore or luminophores are sufficiently distant from the quenching compound that energy transfer and therefore quenching is somewhat or substantially decreased. In yet another embodiment, the compound of the invention is a precursor to

a quenching compound, and the emission of the luminophore or luminophores additionally bound to the conjugated substance is not quenched until conversion of the precursor to a quenching compound.

5 In one embodiment, the conjugated substance (Sc) is a natural or synthetic amino acid (including those that are protected or are substituted by phosphates, carbohydrates, or C<sub>1</sub> to C<sub>22</sub> carboxylic acids), or is a natural or synthetic polymer of amino acids such as a peptide or protein that is optionally derivatized by a chemical protecting group. Preferred conjugates of peptides contain at least five amino acids, more preferably 5 to 36 amino acids. Preferred peptides include, but are not limited to, neuropeptides, cytokines, toxins, protease substrates, and protein kinase substrates.

10 Preferred protein conjugates include enzymes, antibodies, lectins, glycoproteins, histones, albumins, lipoproteins, avidin, streptavidin, protein A, protein G, phycobiliproteins and other fluorescent proteins (such as green fluorescent protein), hormones, toxins and growth factors. Typically, the conjugated protein is an antibody, an antibody fragment, an avidin, a streptavidin, a toxin, a lectin, a hormone, or a growth factor. Typically where the conjugated substance is a toxin, it is a

15 neuropeptide or a phallotoxin, such as phalloidin. In another embodiment, the conjugated substance is a polypeptide or protein that is a substrate for an exopeptidase or an endopeptidase. Where the conjugated substance is a peptidase substrate that is simultaneously labeled with a luminophore or luminophores, the action of the peptidase enzyme cleaves the polypeptide, resulting in restoration of luminescence.

20 In another embodiment, the conjugated substance (Sc) is a natural or synthetic nucleic acid base, nucleoside, nucleotide or a nucleic acid polymer, including those that are protected, or modified to possess an additional linker or spacer for attachment of the compounds of the invention, such as an alkynyl linkage (US Pat. 5,047,519), an aminoallyl linkage (US Pat. 4,711,955) or other linkage. Preferably, the conjugated nucleotide is a nucleoside triphosphate or a deoxynucleoside triphosphate

25 or a dideoxynucleoside triphosphate.

Preferred nucleic acid polymer conjugates are labeled, single- or multi-stranded, natural or synthetic DNA or RNA, DNA or RNA oligonucleotides, or DNA/RNA hybrids, or incorporate an unusual linker such as morpholine derivatized phosphates (AntiVirals, Inc., Corvallis OR), or peptide nucleic acids such as *N*-(2-aminoethyl)glycine units. When the nucleic acid is a synthetic

30 oligonucleotide, it typically contains fewer than 50 nucleotides, more typically fewer than 25 nucleotides. Larger nucleic acid polymers are typically prepared from labeled nucleotides or oligonucleotides using oligonucleotide-primed DNA polymerization, such as by using the polymerase chain reaction or through primer extension, or by terminal-transferase catalyzed addition of a labeled nucleotide to a 3'-end of a nucleic acid polymer. Typically, the compound of the invention is

35 attached via one or more purine or pyrimidine bases through an amide, ester, ether or thioether bond; or is attached to the phosphate or carbohydrate by a bond that is an ester, thioester, amide, ether or thioether. Alternatively, the compound of the invention is bound to the nucleic acid polymer by chemical post-modification, such as with platinum reagents, or using a photoactivatable molecule

such as a conjugated psoralen. In one embodiment, the quenching moiety is attached to the nucleotide, oligonucleotide or nucleic acid polymer via a phosphoramidite reactive group, resulting in a phosphodiester linkage. Alternatively, a conjugate of the invention is simultaneously labeled with a hapten such as biotin, digoxigenin, or 2,4-dinitrophenyl. Nucleotide conjugates of the invention are readily incorporated by a DNA polymerase.

In another embodiment, the conjugated substance (Sc) is a carbohydrate that is typically a natural or synthetic polysaccharide, such as a dextran, FICOLL, heparin, glycogen, amylopectin, mannan, inulin, starch, agarose and cellulose. The carbohydrates of the invention are optionally derivatized by protecting groups such as those used in carbohydrate synthesis. The conjugated carbohydrates are optionally further substituted by one or more luminophores, that are optionally the same or different. Alternatively, the carbohydrate is a polysaccharide that is a lipopolysaccharide. Preferred polysaccharide conjugates are dextran, starch, or FICOLL conjugates.

In another embodiment, the conjugated substance (Sc), is a lipid (typically having 6–60 carbons), including glycolipids, phospholipids, sphingolipids, glycerides, and steroids. Where the lipid is a phospholipid, the compound of the invention is preferably incorporated in the polar head group of the lipid. Alternatively, the conjugated substance is a lipid assembly, such as a liposome or a lipid droplet. Such a lipid assembly optionally comprises one or more additional luminophores.

Other conjugates of non-biological materials include conjugates of organic or inorganic polymers, polymeric films, polymeric wafers, polymeric membranes, polymeric particles, polymeric microparticles including magnetic and non-magnetic microspheres, conducting and non-conducting metals and non-metals, and glass and plastic surfaces and particles, which are optionally also conjugated to one or more luminophores, which may be the same or different. Conjugates are optionally prepared by copolymerization of a compound of the invention that contains an appropriate functionality while preparing the polymer, or by chemical modification of a polymer that contains functional groups with suitable chemical reactivity. Other types of reactions that are useful for preparing conjugates of polymers include catalyzed polymerizations or copolymerizations of alkenes, such as acrylamides, and reactions of dienes with dienophiles, transesterifications or transaminations. The compounds of the invention are optionally incorporated in polymers by polymerization of the monomer in the presence of the compound of the invention, resulting in non-covalent entrapment of the compound of the invention within the polymer matrix. In another embodiment, the conjugated substance is a glass or silica, which may be formed into an optical fiber or other structure.

#### Applications

The term "quenching compound" is used herein to refer to all aspects of the claimed quenching xanthenes, including rhodamine dyes and rhodol dyes, while "compound of the invention" is used to refer to both the quenching compounds of the invention and their colorless precursors.

In one aspect of the invention, the quenching compounds of the invention are useful simply as colorimetric labels for a conjugated substance. The compounds of the invention typically have large extinction coefficients, and thereby permit the detection of the quenching compound-conjugated substance by virtue of the visible light absorption of the quenching compound.

5       The quenching compounds of the present invention accept energy from a wide variety of luminophores, provided that the quenching compound and the luminophore are in sufficiently close proximity for quenching to occur, and that at least some spectral overlap occurs between the emission wavelengths of the luminophore and the absorption band of the quenching compound. This overlap may occur with emission of the donor occurring at a lower or even higher wavelength  
10       emission maximum than the maximal absorbance wavelength of the quenching compound, provided that sufficient spectral overlap exists. Energy transfer may also occur through transfer of emission of the donor to higher electronic states of the acceptor, such as from tryptophan residues of proteins to the weaker absorption bands between 300 and 350 nm typical of the dyes in the ultraviolet region. Preferably, the quenching compound of the invention is only dimly fluorescent, or essentially  
15       nonfluorescent, so that energy transfer results in little or no fluorescence emission.

In one aspect of the invention, the quenching compound of the invention has a fluorescence quantum yield of less than about 0.05 (see, for example, Table 3). In another aspect of the invention, the quenching compound of the invention has a fluorescence quantum yield of less than about 0.01. In yet another aspect of the invention, the quenching compound of the invention has a fluorescence  
20       quantum yield of less than about 0.005.

Any luminophore with sufficient spectral overlap with a quenching compound of the instant invention, as calculated above, is a suitable donor for the applications of the invention, other factors being equal. The greater the degree of overlap, the greater the overall quenching observed. While fluorescent dyes are preferred for energy transfer applications, any emission that generates light  
25       having sufficient spectral overlap with the quenching compounds of the invention is also useful, such as chemiluminescence, or phosphorescence, whether by FRET or by triplet state to singlet state transfer.

It should be readily appreciated that the degree of energy transfer during FRET, and therefore quenching, is highly dependent upon the separation distance between the luminophore and  
30       the quenching compound. In molecular systems, a change in luminescence quenching typically correlates well with a change in the separation distance between the luminophore molecules and the quenching compound molecules. Assays that detect such changes in luminescence are therefore useful for the detection of a great many structural changes, such as changes in molecular conformation, assembly of structures, or degradation of structures.

35       While FRET is the most common mechanism for quenching of fluorescence to occur, any combination of molecular orientation and spectral coincidence that results in quenching of luminescence is a useful mechanism for quenching by the quenching compounds of the invention, as described herein. For example, efficient quenching can occur even in the absence of spectral overlap

if the luminophore and the quenching compound are sufficiently close together to form a ground-state complex (as described in Tyagi et al., NATURE BIOTECHNOLOGY 16, 49 (1998)).

A wide variety of chemically reactive fluorescent dyes that would be suitable luminophores (donors) are already known in the art (see for example MOLECULAR PROBES HANDBOOK OF  
5 FLUORESCENT PROBES AND RESEARCH CHEMICALS, Sixth Ed., Richard P. Haugland, ed. (1996), in particular Chapters 1-3; BIOPROBES 26 (October 1997); BIOPROBES 27 (February 1998); BIOPROBES 28 (May 1998); BIOPROBES 29 (November 1998); BIOPROBES 30 (January 1999) BIOPROBES 31 (May 1999); BIOPROBES 32 (December 1999); and BIOPROBES 33 (February 2000). The spectral properties of candidate dyes in solution or when conjugated to a  
10 selected biomolecule are known or are readily measured using a spectrofluorometer.

Typically, where the luminophore is a fluorophore, it is a fluorescent aromatic or heteroaromatic compound that is a pyrene, an anthracene, a naphthalene, an acridine, a stilbene, an indole or benzindole, an oxazole or benzoxazole, a thiazole or benzothiazole, a 4-amino-7-nitrobenz-2-oxa-1,3-diazole (NBD), a cyanine, a carbocyanine, a carbostyryl, a porphyrin, a salicylate, an  
15 anthranilate, an azulene, a perylene, a pyridine, a quinoline, a coumarin (including hydroxycoumarins and aminocoumarins and fluorinated derivatives thereof (as described in U.S. Patent No. 5,830,912 to Gee et al. (1998)), a polyazaindacene (such as 4-bora-3a,4a-diaza-s-indacene as described in US Patents 4,774,339 to Haugland, et al. (1988); 5,187,288 to Kang, et al. (1993); 5,248,782 to Haugland, et al. (1993); 5,274,113 to Kang, et al. (1993); and 5,433,896 to Kang, et  
20 al.(1995)), a xanthene, an oxazine or a benzoxazine, a carbazine (US Patent 4,810,636 to Corey (1989)), or a phenalenone or benzphenalenone (US Patent 4,812,409 Babb et al. (1989)). The donor dye is optionally an organic molecule that is a fluorophore, or a fluorescent protein such as a phycobiliprotein or "green fluorescent protein". Preferably, the donor dye is a carbazine, an oxazine, a coumarin, a pyrene, a xanthene, a naphthalene, a phenalenone, or a 4-bora-3a,4a-diaza-s-indacene.  
25 As used herein, oxazines include resorufins, aminooxazinones, diaminoxazines, and their benzo-substituted analogs. Preferred chemiluminescent dyes include luminols, isoluminols, luciferins, acridinium esters, or dioxetanes.

Where the synthetic dye is a xanthene, the synthetic dye is optionally a fluorescein, a rhodol (US Patent 5,227,487 to Haugland, et al. (1993)), or a rhodamine. As used herein, fluorescein  
30 includes benzo- or dibenzofluoresceins, seminaphthofluoresceins, or naphthofluoresceins. Similarly, as used herein rhodol includes seminaphthorhodafuors (US Patent 4,945,171 to Haugland, et al. (1990)). Fluorinated xanthene dyes have also been described previously (Int. Publ. No. WO 97/39064, Molecular Probes, Inc. (1997)). Sulfonated pyrenes, coumarins, carbocyanines, and xanthene dyes have been described previously (U.S. Patent No. 5,132,432 to Haugland et al., (1992);  
35 U.S. Patent No. 5,696,157 to Wang et al. (1997); U.S. Patent No. 5,268,486 patent to Waggoner et al. (1993); and International Publication No. WO 99/15517 by Mao et al. (1999)).

### Method of Use

The quenching compounds of the invention are useful in any application where energy transfer from a luminescent donor to a non-fluorescent acceptor has previously been described, provided that some spectral overlap exists between the emission of the donor dye and the absorbance of the quenching compound of the invention. Typically, the quenching compounds are used in combination with a luminophore in a method that detects a change in separation distance between the luminophore and the quenching compound.

The quenching compounds of the invention are generally utilized by labeling a substance or sample of interest under conditions selected so that illumination of the sample with an appropriate wavelength of light results in a detectable optical response. In one embodiment, the quenching compounds of the invention are utilized as colorimetric labels, such that the detectable optical response is an absorption of illumination energy. In another embodiment the quenching compound accepts energy from a donor, such that the detectable optical response is quenching of the luminescence of the donor.

The labeled substance may be utilized in a homogenous solution assay, where specific spatial resolution is not required. In this case the loss of, or restoration of, luminescence in the sample is detected. Alternatively, the quenching compound forms a covalent or non-covalent association or complex with an element of the sample where a luminescent component is present or is added, resulting in a luminescence response if quenching is not occurring, or the degree of quenching may be correlated with a characteristic of the sample. Typically, a stained sample is illuminated and observed and the degree of quenching exhibited is compared to a luminescence standard. The luminescence standard may be a fluorescent dye such as the fluorophore, a luminescent particle (including fluorescent microspheres), a calibration curve, or any other standard that can be used to calibrate luminescence signal intensity as well known in the art.

Typically, the method of the invention comprises the steps of

- a) illuminating the molecular system under study;
- b) detecting the luminescence response of the system, which yields information as to the separation distance of one or more luminophore donors and quenching compound acceptors;
- c) exposing the molecular system to an environmental condition sufficient to change the separation distance, or thought to be sufficient to change the separation distance;
- d) illuminating the molecular system again;



e) detecting the luminescence response of the molecular system again; and

f) comparing the first detected luminescence response to the second detected luminescence response, in order to determine a detectable difference in the detected luminescence before and after the exposure to the selected environmental condition. The detected change in the luminescence of the molecular system then correlates with any changes that occurred in the separation distance between the luminophores and the quenching compounds, typically in response to the selected environmental condition.

As discussed in greater detail below, the environmental condition of the instant method may include, without limitation, the presence of a particular enzyme, the presence of a complementary specific binding pair member, a change in pH, or a change in sample temperature.

#### *Illumination and detection*

Typically, changes in luminescence quenching are detected by methods well known in the art for standard luminescence assays. Sample luminescence, if present, is typically detected by illumination of the sample with a light source capable of producing light that is absorbed at or near the wavelength of maximum absorption of the donor dye, and luminescence is detected at a wavelength longer than the excitation wavelength, typically near the emission maximum. The sample luminescence may be detected using methods and instrumentation well known in the art.

#### *Applications*

As described above, the method of the instant invention is typically useful for detection of changes in separation distance between a luminophore donor and a quenching compound acceptor.

Any assay that relies upon the measurement of the proximity of luminophores and quenching compounds in a system may be carried out using the method of the instant invention. The method of the instant invention is typically utilized to detect and/or quantify the convergence or divergence of the luminophore donor and quenching compound acceptor. By convergence is meant a decrease in the average separation distance between the luminophore and the quenching compound. By divergence is meant an increase in the average separation distance between the luminophore and the quenching compound. In yet another embodiment, the method of the invention is utilized to detect a conformation change in a molecule, macromolecule or structure (optionally convergence or divergence).

*Detection of Structural Assembly*

In one embodiment, the luminescence of a luminophore becomes quenched upon being placed in close proximity to a quenching compound of the invention (thereby decreasing the separation distance). The following systems, among others, can be analyzed using energy transfer pairs to  
 5 detect and/or quantify structural assembly by measuring convergence of the donor and acceptor:

- protein subunit assembly
- enzyme-mediated protein assembly
- 10 • molecular dimensions of proteins
- membrane-protein interactions
- protein-protein interactions
- protein-protein-nucleic acid complex assembly
- receptor/ligand interactions
- 15 • immunoassays
- nucleic acid hybridization
- quantitative detection of specific DNA sequence amplification
- detection of DNA duplex winding
- nucleic acid-protein interactions
- 20 • nucleic acid-drug interactions
- primer extension assays for mutation detection
- reverse transcriptase assay
- strand exchange in DNA recombination reactions
- membrane fusion assays
- 25 • transmembrane potential sensing
- ligation assays

In particular, specific binding pair members labeled with a quenching compound are typically used as probes for the complementary member of that specific binding pair, by methods  
 30 well known in the art. The complementary member is typically labeled with a luminescent label, and association of the two members of the specific binding pair results in luminescence quenching. This assay is particularly useful in nucleic acid hybridization assays, evaluation of protein-nucleic acid interaction, and in selected standard immunoassays. In one embodiment, a loss of luminescence indicates the association of an enzyme with an enzyme substrate, agonist or antagonist, such that  
 35 the luminophore on one is brought into close proximity to a quenching compound on the other. Selected preferred specific binding pair members are proteins that bind non-covalently to low molecular weight ligands (including biotin), oligonucleotides, and drug-haptens. Representative specific binding pairs are shown in Table 1.

Table 1. Representative Specific Binding Pairs

antigen	antibody
biotin	avidin, streptavidin, anti-biotin
folate	folate-binding protein
IgG*	protein A or protein G
drug	drug receptor
toxin	toxin receptor
carbohydrate	lectin or carbohydrate receptor
peptide	peptide receptor
protein	protein receptor
peptide nucleic acid	complementary strand
enzyme substrate	enzyme
DNA (RNA)	cDNA (cRNA)†
hormone	hormone receptor
ion	chelator

\* IgG is an immunoglobulin

† cDNA and cRNA are complementary strands used for hybridization

Alternatively, a monomer, labeled with a quenching compound, is incorporated into a polymer labeled with a luminophore, resulting in quenching of luminescence. In particular, a quenching compound-labeled nucleotide can be incorporated via the polymerase chain reaction into a double stranded DNA molecular that is labeled with a luminophore.

#### *Detection of Structural Disassembly*

In another embodiment of the method of the invention, the disassembly, cleavage or other degradation of a molecular structure is detected by observing the partial or complete restoration of luminescence of a luminophore donor. Typically, the initially quenched luminescence of a luminophore associated with the structure becomes dequenched upon being released from the constraint of being in close proximity to a quenching compound of the invention. The quenching compound is optionally associated with the same molecular structure as the luminophore, or the donor and acceptor are associated with adjacent but distinct subunits of the structure. The following systems, among others, can be analyzed using energy transfer pairs to detect and/or quantify structural disassembly:

- detection of protease activity using fluorogenic substrates (for example HIV protease assays)

- detection of enzyme-mediated protein modification (e.g. cleavage of carbohydrates/fatty acids, phosphates, prosthetic groups)
- immunoassays (via displacement/competitive assays)
- detection of DNA duplex unwinding (e.g. helicase/topoisomerase/gyrase assays)
- 5 • nucleic acid strand displacement
- ds DNA melting
- nuclease activity
- lipid distribution and transport
- TAQMAN assays

Structure disassembly is typically detected by observing the partial or complete restoration of luminescence, as a conjugated substance is exposed to a degradation conditions of interest for a period of time sufficient for degradation to occur. A restoration of luminescence indicates an increase in separation distance between the luminophore and quenching compound, and therefore a degradation of the conjugated substance. If the detectable difference in luminescence is detected as the degradation proceeds, the assay is a continuous assay. Since most enzymes show some selectivity among substrates, and as that selectivity can be demonstrated by determining the kinetic differences in their hydrolytic rates, rapid testing for the presence and activity of the target enzyme is provided by the enhancement of luminescence of the labeled substrate following separation from the quenching compound.

In another embodiment of the invention, a single-stranded oligonucleotide signal primer is labeled with both a quenching compound and a fluorescent donor dye, and incorporates a restriction endonuclease recognition site located between the donor dye and the quenching compound. The single-stranded oligonucleotide is not cleavable by a restriction endonuclease enzyme, but upon binding to a complementary (target) nucleic acid, the resulting double stranded nucleic acid is cleaved by the enzyme and the decreased quenching is used to detect the presence of the complementary nucleic acid (U.S. Patent No. 5,846,726 to Nadeau et al., (1998)).

In yet another embodiment of the invention, structural disassembly can be detected by the quenching of luminescence. In this embodiment, the action of an oxidative enzyme on a colorless precursor results in the generation of a quenching compound of the invention. The newly generated quenching compound then quenches the luminescence of a luminophore if it is in sufficiently close proximity, indicating the presence and/or activity of the enzyme. Alternatively, where  $R^7$  is selected so as to make the colorless precursor an enzyme substrate (where  $R^7$  is a monovalent radical formally derived by removing a hydroxy group from a carboxylic acid, a sulfonic acid, a phosphoric acid, or a mono- or polysaccharide), the generation of the quenching compounds of the invention indicates the presence of the particular enzyme. In another embodiment,  $R^7$  is a photolabile caging group, including but not limited to *o*-nitroarylmethine derivatives, 2-methoxy-5-nitrophenyl derivatives, or desyl derivatives.

A single nucleotide polymorphism (SNP) can be detected through the use of sequence-specific primers, by detection of melt temperatures of the double stranded nucleic acid. In this aspect, the complementary or substantially complementary strands are labeled with a quenching compound and a luminophore donor, respectively, and dissociation of the two strands (melting) is detected by the restoration of luminescence of the donor.

In yet another example of a divergence assay, the rupture of a vesicle containing a highly concentrated solution of luminophores and quenching compounds is readily detected by the restoration of luminescence after the vesicle contents have been diluted sufficiently to minimize quenching.

#### *Detection of conformation changes*

In this embodiment, the quenching compound and the fluorescent donor are present on the same or different substances, and a change in the three-dimensional structural conformation of one or more components of the assay results in either luminescence quenching or restoration of luminescence, typically by substantially decreasing or increasing the separation distance between the quenching compound and a luminophore. The following systems, among others, can be analyzed using energy transfer pairs to detect and/or quantify conformation changes :

- protein conformational changes
- protein folding
- structure and conformation of nucleic acids
- drug delivery
- antisense oligonucleotides
- cell-cell fusion (e.g. via the diffusion apart of an initial donor-quenching compound pair)

By conformation change is meant, for example, a change in conformation for an oligonucleotide upon binding to a complementary nucleic acid strand. In one such assay, labeled oligonucleotides are substantially quenched when in solution, but upon binding to a complementary strand of nucleic acid become highly fluorescent (so-called "Molecular Beacons", as described in European patent application EP 0 745 690, by Tyagi et al (1996)). Another example detects the change in conformation when an oligonucleotide that has been labeled at its ends with a quenching compound and a luminophore, respectively, loses its G-quartet conformation upon hybridization to a complementary sequence, resulting in decreased luminescence quenching (U.S. Patent No. 5,691,145 to Pitner et al. (1997)). Alternatively, the binding of an enzyme substrate within the active site of a labeled enzyme may result in a change in tertiary or quaternary structure of the enzyme, with restoration or quenching of luminescence.

*Additional Detection Reagents*

When used in complex systems, especially in biological cells, the assays of the instant invention are optionally combined with the use of one or more additional detection reagents, such as an antibody, or a stain for another component of the system such as a nucleic acid stain, an organelle stain, a metal ion indicator, or a probe to assess viability of the cell. The additional detection reagent is optionally a fluorescent reagent exhibiting a color that contrasts with the donor dye present in the assay, or is a label that is detectable by other optical or non-optical properties.

10 Kits

One aspect of the instant invention is the formulation of kits that facilitate the practice of the methods of the invention, as described above. The kit of the invention comprises a quenching compound of the invention, or colorless quenching compound precursor of the invention, typically present conjugated to a nucleotide, oligonucleotide, nucleic acid polymer, peptide, or protein. Typically, the kit further comprises one or more buffering agents, typically present as an aqueous solution. The kits of the invention optionally further comprise additional detection reagents, a purification medium for purifying the resulting labeled substance, luminescence standards, enzymes, enzyme inhibitors, organic solvent, or instructions for carrying out an assay of the invention.

20 In one embodiment, the kit comprises a quenching compound of the invention and a luminescent donor. The quenching compound and luminescent donor are optionally each attached to a conjugated substance, or present as discrete solutions of free compounds. Such a kit would be useful for the detection of cell-cell fusion

In another embodiment, the kit comprises a quenching compound and a luminescent donor, each conjugated to a complementary member of a specific binding pair. In this aspect of the invention, binding of the two specific binding pair members results in quenching of luminescence, and the kit is useful for the detection of competitive binding to one or the other specific binding pair members, or for the detection of an environmental condition or component that either facilitates or inhibits binding of the specific binding pair members.

30 In another embodiment, the kit comprises a conjugate of a quenching compound and a conjugate of a luminescent donor, wherein the conjugates are selected such that the action of a particular enzyme results in covalent or noncovalent association of the two conjugates, resulting in quenching of fluorescence. Where the conjugated substances are nucleotides, oligonucleotides or nucleic acid polymers, the kit is useful for the detection of, for example, ligase, telomerase, helicase, topoisomerase, gyrase, DNA/RNA polymerase, or reverse transcriptase enzymes.

35 In another embodiment, the kit comprises a biomolecule that is covalently labeled by both a quenching compound of the invention and a luminescent donor. In one aspect, the labeled biomolecule exhibits luminescence until a specified environmental condition (such as the presence of

a complementary specific binding pair) causes a conformation change in the biomolecule, resulting in the quenching of luminescence. Alternatively, the biomolecule is initially quenched, and a specified environmental condition (such as the presence of an appropriate enzyme or chemical compound) results in degradation of the biomolecule and restoration of luminescence. Such a kit would be useful for the detection of complementary oligonucleotide sequences, or for the detection of enzymes such as nuclease, lipase, protease, or cellulase.

### Synthesis

Rhodamine dyes are typically prepared by condensation of an appropriate aminophenol with various derivatives of benzoic acid, phthalic acid or phthalic anhydride or sulfobenzoic acid or its anhydride. In the case of the quenching compounds of the invention, the aminophenol is typically an N-aryl or N-heteroaryl substituted aminophenol. Useful derivatives of benzoic acid include, without limitation, phthalic anhydride, trimellitic anhydride, nitrophthalic anhydride, polyhalogenated phthalic anhydrides, o-sulfobenzoic anhydride, sulfoterephthalic acid, or benzaldehydes or aliphatic dicarboxylic acids or anhydrides such as a succinic anhydride or a glutaric anhydride. This condensation reaction occurs in the presence or absence of various acid catalysts (such as zinc chloride, p-toluenesulfonic acid, sulfuric acid, or methanesulfonic acid). An aqueous workup, typically followed by column chromatography, yields the desired xanthylium dye.

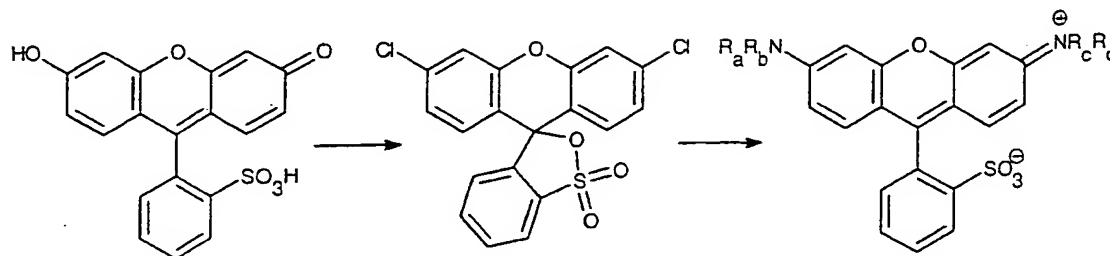
If an unsymmetric rhodamine dyes is desired, the condensation can be performed using one equivalent each of the appropriate substituted or unsubstituted aminophenol with one equivalent of a different aminophenol and with one equivalent of the appropriate phthalic acid derivative or benzaldehyde (as listed above) using acid catalysis (as in Khanna et al., U.S. Patent No. 4,439,359 (1984) and Haugland et al., U.S. Patent No. 5,227,487 (1993)). The desired asymmetric rhodamine dye is separated from any unwanted symmetric dye side-product(s) using crystallization or chromatographic techniques well-known in the art.

Unsymmetric rhodamine dyes can also be constructed in a stepwise fashion: A selected aminophenol is condensed with one equivalent of the appropriate benzoic or phthalic acid derivative or benzaldehyde. The resulting benzophenone derivative is typically isolated, purified and then condensed with one equivalent of a different aminophenol, yielding the asymmetric dye.

Rhodol dyes are prepared by condensation of one equivalent each of the appropriate substituted or unsubstituted resorcinol with one equivalent of the desired N-aryl or N-heteroaryl aminophenol and with one equivalent of the appropriate phthalic acid derivative or benzaldehyde using acid catalysis (U.S. Patent No. 5,227,487 to Haugland et al. (1993)).

Aryl and heteroaryl substituted xanthylium dyes are also readily prepared by displacement of the chloro groups from 3,6-dichlorofluoran or 3,6-dichlorosulfonefluoran using an appropriately substituted amine (Equation 1; U.S Patent No. 4,258,118 and German Patent 24 60 491). 5-

Carboxy-3,6-dichlorosulfonefluoran is readily converted to a 5-carboxy-3,6-diarylamino derivative using a similar methodology.



Equation 1

A variety of aryl-substituted rhodamines may be prepared using this method (Example 1).

The post-condensation modification of rhodamine and rhodol dyes is well known in the chemical arts. For example, the xanthenone portion of the dye can be halogenated by treatment with the appropriate halogenating agent, such as liquid bromine. Xanthenes containing unsaturated fused rings can be hydrogenated to the saturated derivatives. The Q moieties are also subject to synthetic modification, either after the condensation reaction, or by modification of aryl and heteroaryl moieties on synthetic precursors.

When trimellitic anhydride or its derivatives is used in the dye synthesis, two isomeric carboxylates are typically formed. These isomers are separated or, in most cases, used as the mixture of isomers. The reduced derivatives of xanthylium dyes are prepared by chemical or electrochemical reduction of the xanthenone portion, such as with zinc dust or borohydride in organic solvents. Sulfonation of xanthylium dyes on the xanthene ring is typically carried out by stirring the dye in fuming sulfuric acid (20-30% SO<sub>3</sub> content) or concentrated sulfuric acid at an appropriate temperature. In some cases, sulfonation on aryl or heteroaryl moieties is possible under these conditions.

The selection of an appropriate polyhalogenated phthalic acid derivative or benzaldehyde in the condensation of the xanthylium dye results in a dye having a di-, tri-, tetra- or pentachlorinated or di-, tri-, tetra- or pentafluorinated phenyl ring at the 9-position. Some of these polyhaloaryl substituted dyes have been shown to react with thiols via a displacement reaction, and thereby provide a facile method of introducing additional reactive groups (Gee, et al. TET. LETT. 37, 7905 (1996)).

The reduced (e.g. dihydrorhodamine) and oxidized versions of the dyes of the invention are freely interconvertible by well-known oxidation or reduction reagents, including borohydrides, aluminum hydrides, hydrogen/catalyst, and dithionites. A variety of oxidizing agents mediate the oxidation of dihydrorhodamines, including molecular oxygen in the presence or absence of a catalyst, nitric oxide, peroxyntirite, dichromate, triphenylcarbenium and chloranil. The dihydrorhodamines



are also oxidized by enzyme action, including horseradish peroxidase in combination with peroxides or by nitric oxide.

In general, the preparation of dye conjugates using reactive dyes is well documented, e.g. by R. Haugland, MOLECULAR PROBES HANDBOOK OF FLUORESCENT PROBES AND RESEARCH CHEMICALS, Chapters 1-3 (1996); Brinkley, BIOCONJUGATE CHEM., 3, 2 (1992); Haugland, "Coupling of Monoclonal Antibodies with Fluorophores", METH. MOL. BIOL. Vol. 45, 205). Conjugates typically result from mixing appropriate reactive dyes and the substance to be conjugated in a suitable solvent in which both are soluble. For those reactive dyes that are photoactivated, conjugation requires illumination of the reaction mixture to activate the reactive dye.

By selection of the appropriate reactive functional group, substances having free amine, thiol, aldehyde or ketone groups are readily coupled to the quenching compounds of the invention by means well known in the art, as described below:

A 5-40 fold molar excess of a succinimidyl ester derivative (such as Compound 5, 8, 11, 13, 24, or 26) may be coupled to an aliphatic amine-modified nucleotide or oligonucleotide in a pH 8.3 buffer (borate or bicarbonate buffer) for greater than 1 hour. The products are purified by HPLC and characterized by their absorption spectra. Succinimidyl ester derivatives may also be coupled to amine-containing peptides or proteins, or to amino saccharides or polysaccharides in a suitable buffer such as borate or bicarbonate buffer and purified either by HPLC or size exclusion chromatography. Conjugates are characterized for their degree of substitution by spectroscopic methods.

Thiolated biomolecules are typically conjugated to thiol-reactive dyes such as Compound 22 at a pH less than 8. Aldehyde- or ketone-containing molecules are typically modified by an amine-, hydrazine- or hydroxylamine-containing dye of the invention (for example Compounds 21 and 30), sometimes followed by chemical reduction of the resulting imine, such as by a borohydride. Low molecular weight molecules are typically prepared in an organic solvent or water, purified chromatographically and characterized for purity by HPLC.

Oligonucleotides conjugated to both a donor dye and a quenching acceptor are readily prepared, preferably by some combination of amine-reactive, thiol-reactive or phosphoramidite derivatives (such as Compound 3). Typically one of the conjugated dyes is a quenching compound of the invention, and at least one of the other conjugated dyes is intrinsically fluorescent

Peptides for use, in particular, as endoprotease substrates that comprise both a fluorescent donor dye and a quenching compound of the invention are synthesized either by modification of peptides that have suitable reactive sites or by use of a building block such as the quenching compound-lysine conjugate (Compound 25).

The examples below are given so as to illustrate the practice of this invention. They are not intended to limit or define the entire scope of this invention.

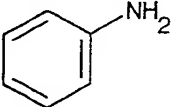
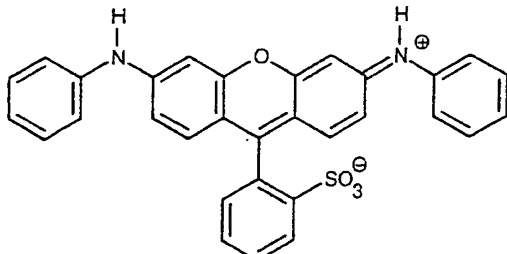
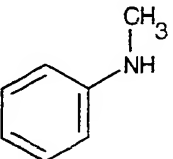
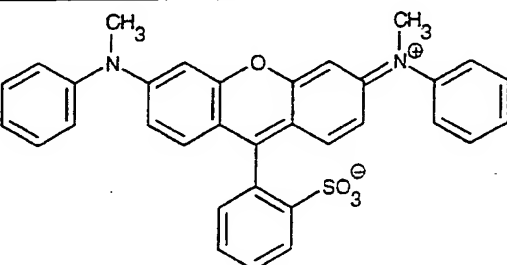
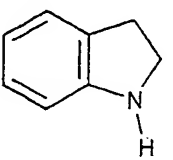
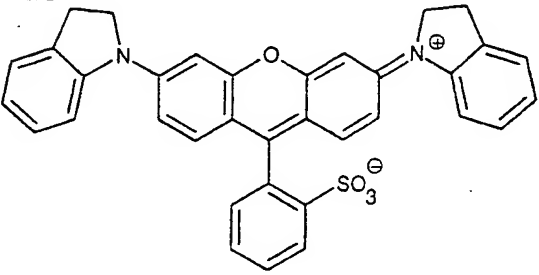
## EXAMPLES

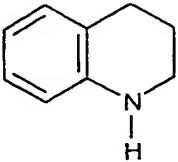
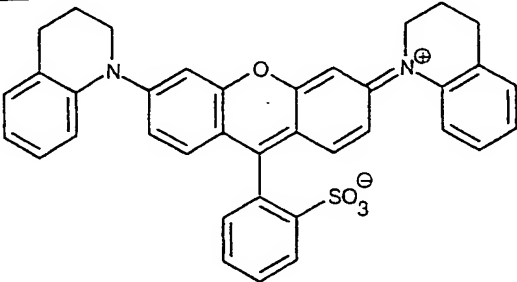
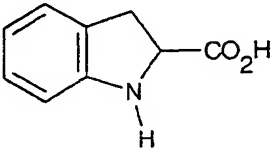
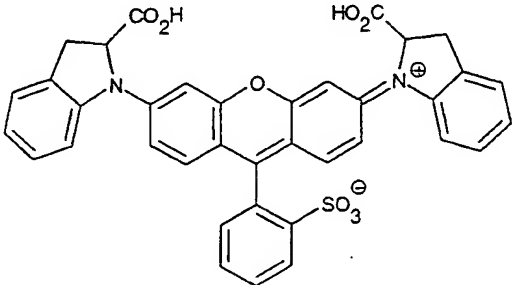
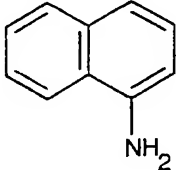
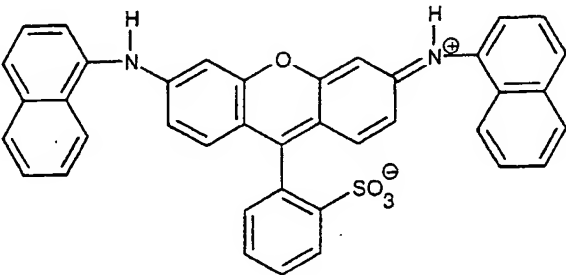
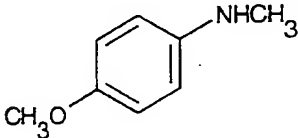
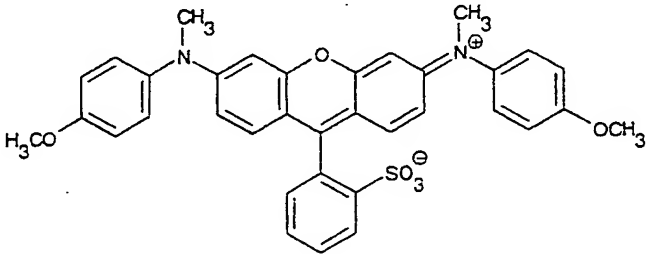
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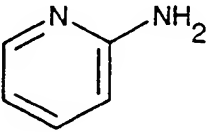
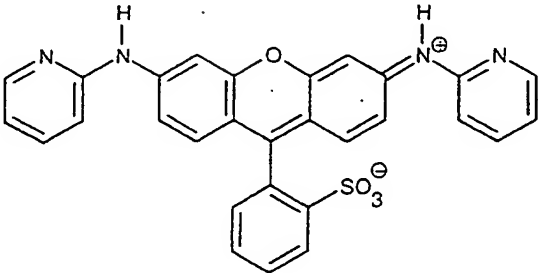
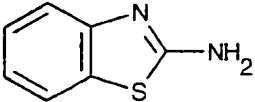
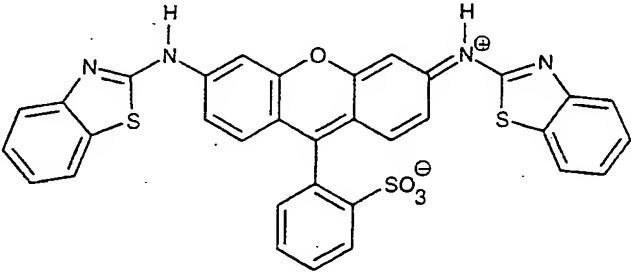
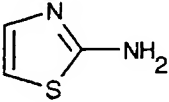
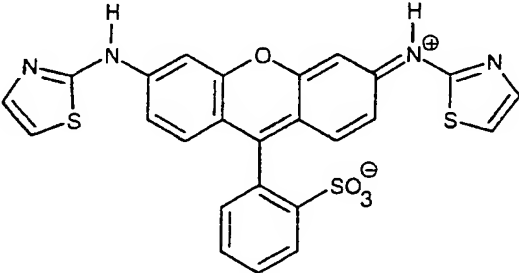
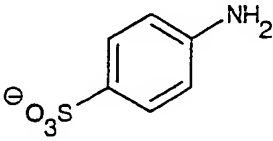
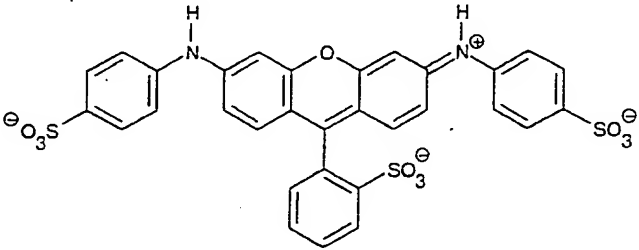
### Example 1. Preparation of aryl-substituted rhodamine sulfonic acid compounds:

Aryl and heteroaryl substituted xanthylium dyes are readily prepared by displacement of the chloro groups from a 3,6-dichlorofluoran or a 3,6-dichlorosulfluoran using the desired substituted  
 10 amine (U.S Patent No. 4,258,118 and German Patent 24 60 491) as shown in Table 2.

Table 2

AMINE USED	RESULTING PRODUCT
	 Compound A
	 Compound B
	 Compound C

	 <p>Compound D</p>
	 <p>Compound E</p>
	 <p>Compound F</p>
	 <p>Compound G</p>

	 <p>Compound H</p>
	 <p>Compound I</p>
	 <p>Compound J</p>
	 <p>Compound K</p>

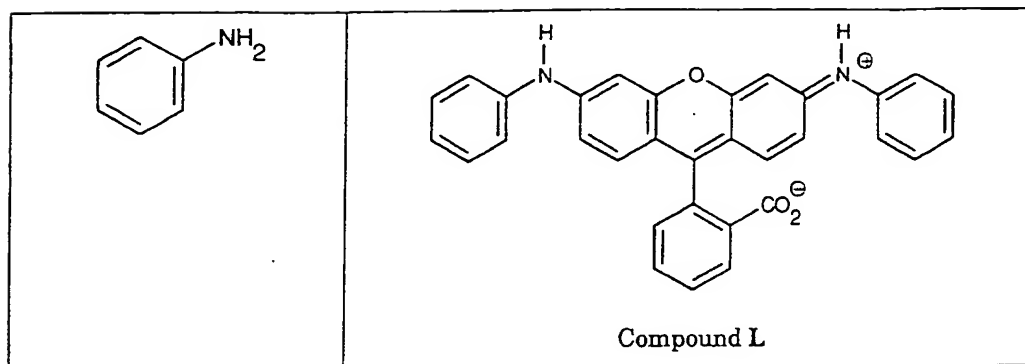


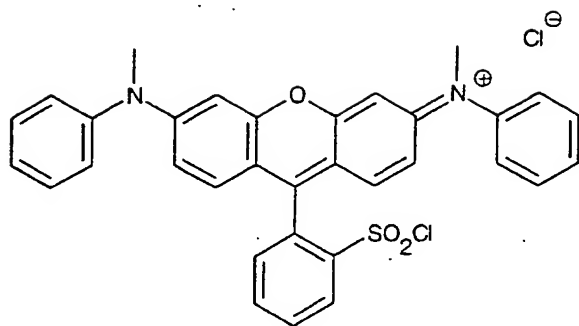
Table 3

Compound	Absorbance $\lambda_{\max}$ (nm)	Quantum Yield
A	553	nd
B	555	nd
C	615	nd
D	629	nd
E	634	nd
F	nd	nd
G	556	nd
H	548	~0.009
I	574	~0.006
J	528	nd
5	561	~0.0009
8	660	~0.0002
10	558	~0.001
13	560	~0.002
16	649	~0.0001
27	553	~0.002
28	560	~0.0002

nd = not determined

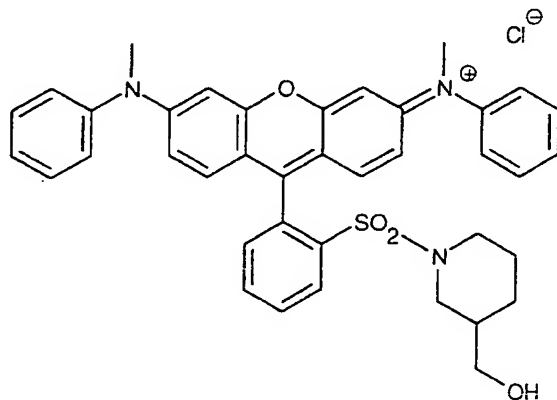
#### 5 Example 2. Preparation of a phosphoramidite derivative (3):

Compound B of Example 1 (2 g) is heated in 20 mL of phosphorous oxychloride at 70 °C for 6 hours. The solution is evaporated under reduced pressure and the residue is dried *in vacuo* for several hours to yield the sulfonyl chloride derivative (Compound 1).



Compound 1

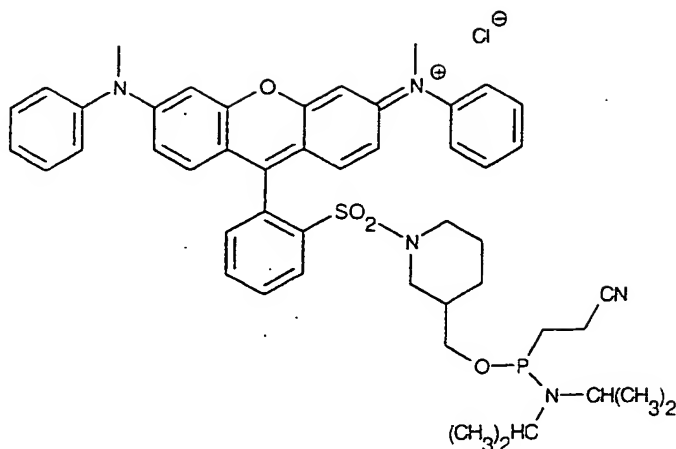
The crude sulfonyl chloride thus generated is stirred in 45 mL of acetonitrile and cooled to 0 °C. To this cold solution is added a mixture of 1.2 g of 3-piperidinemethanol, 2 mL of triethylamine  
 5 in 6 mL of DMF and 10 mL of acetonitrile. After one hour the acetonitrile is evaporated, the reaction mixture is poured into 1 M HCl and extracted with chloroform. Following evaporation, the crude residue is purified on a silica gel column to yield 1.7 g of the methanol adduct 2:



Compound 2

10

To 136 mg of 2 in 2 mL of methylene chloride at 0 °C under nitrogen is added sequentially 0.19 mL of diisopropylethylamine and 0.23 mL of 2-cyanoethyl diisopropylchlorophosphoramidite. The reaction mixture is stirred for 5 minutes then poured onto sodium bicarbonate and extracted with methylene chloride. The methylene chloride is removed under reduced pressure to yield the  
 15 phosphoramidite derivative, 3.



Compound 3

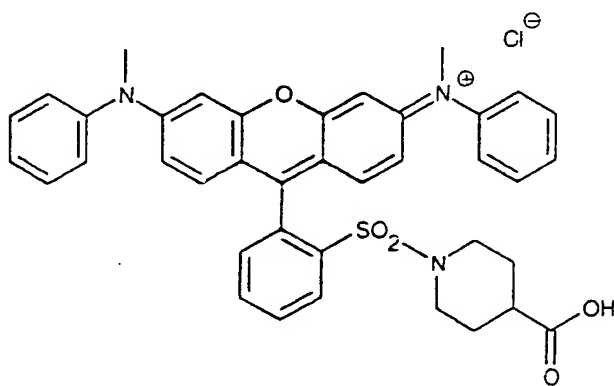
The phosphoramidite derivative is useful for the preparation of quenching compound-labeled oligonucleotides using an automated synthesizer.

5

**Example 3. Preparation of a succinimidyl ester derivative (5):**

Isonipecotic acid (20.5 g) is heated at reflux for 30 minutes in 80 mL of hexamethyldisilazane in the presence of a catalytic amount of concentrated sulfuric acid. Excess hexamethyldisilazane is evaporated and the residue is dissolved in 150 mL of acetonitrile and cooled to 0-5 °C. Triethylamine (23 mL) is added, followed by 29 g of the sulfonyl chloride derivative Compound 1 in 150 mL of acetonitrile to generate Compound 4, which is isolated by evaporation of the solvent, addition of 1 M HCl, extraction into chloroform and evaporation.

10

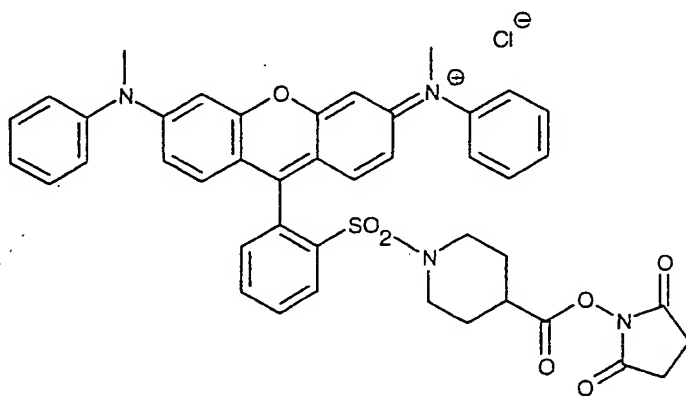


Compound 4

15

To a stirring solution of Compound 4 (2.26 g) in 20 mL of acetonitrile at room temperature are added sequentially 1.1 mL of diisopropylethylamine and 1.7 g of 2-succinimido-1,1,3,3-tetramethyluronium tetrafluoroborate. The resulting mixture is stirred at room temperature for one

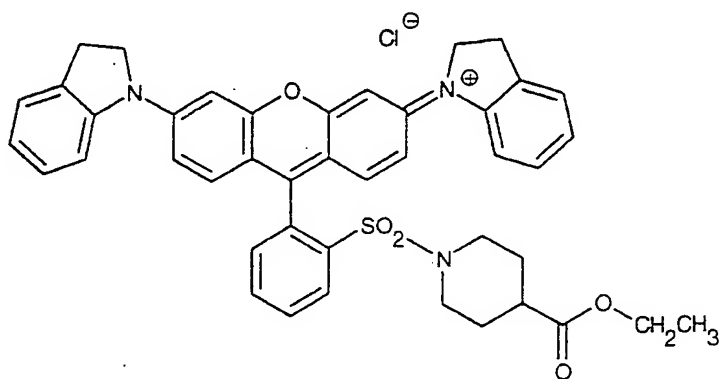
hour. The solution is partitioned between  $\text{CHCl}_3$  and 1 M HCl, and the organic portion is evaporated and recrystallized from  $\text{CH}_3\text{CN}$  and diethyl ether to yield the desired succinimidyl ester, Compound 5.



Compound 5

**Example 4. Preparation of a succinimidyl ester derivative (8):**

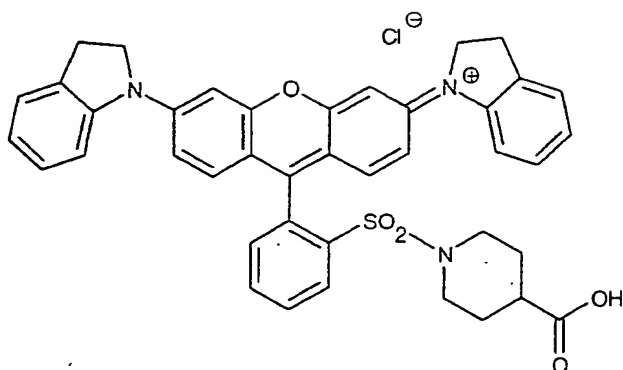
Compound C (Example 1) is treated with phosphorous oxychloride and ethyl isonipecotate following the procedure of Example 2 to the ester Compound 6.



Compound 6

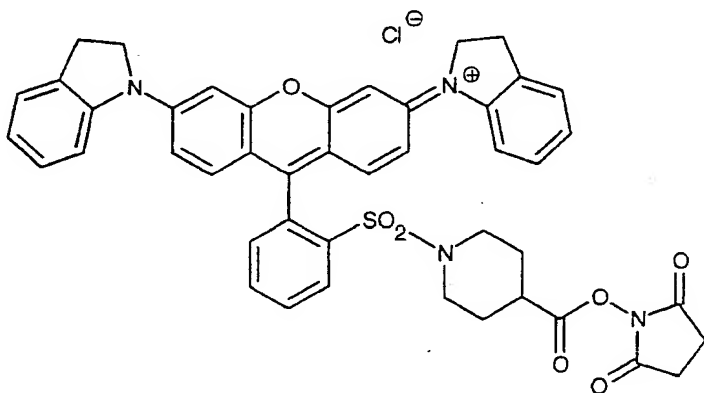
To 0.31 g of ester 6 in 60 mL of methanol is added 1.5 mL of a 10 % NaOH solution and the mixture is heated at 35-40 °C overnight. Another 2 mL of 10% NaOH is introduced and heating is continued for another 20 hours. The reaction mixture is cooled to room temperature, 120 mL of 1 M HCl is added and after another hour the product is filtered to give product 7.





Compound 7

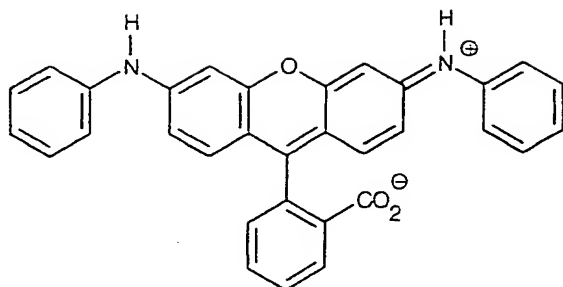
To 98 mg of acid 7 in 1.5 mL of DMF is added 0.07 mL of diisopropylethylamine and 66 mg of 2-succinimido-1,1,3,3-tetramethyluronium tetrafluoroborate. The reaction is worked up with chloroform and 1 M HCl and the organic extracts are dried over magnesium sulfate. The crude material is dissolved in 2 mL of acetonitrile. Ethyl acetate (8 mL) is added dropwise and after stirring overnight the solution is filtered to yield 80 mg of product 8.



Compound 8

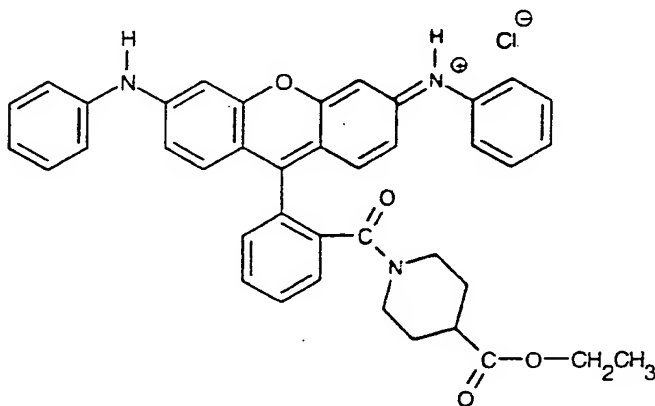
**Example 5. Preparation of a succinimidyl ester derivative (11):**

A mixture of 5.52 g of 3-hydroxydiphenylamine, 2.21 g of phthalic anhydride, and 2.04 g of  $\text{ZnCl}_2$  is heated at  $180^\circ\text{C}$  for 1.5 hour. A mixture of methanol (150 mL) and water (50 mL) is added and the mixture is heated at reflux overnight. The solid is filtered and then refluxed again in 100 mL of methanol and 50 mL of water for 3 hours. The mixture is filtered to yield the acid 9.



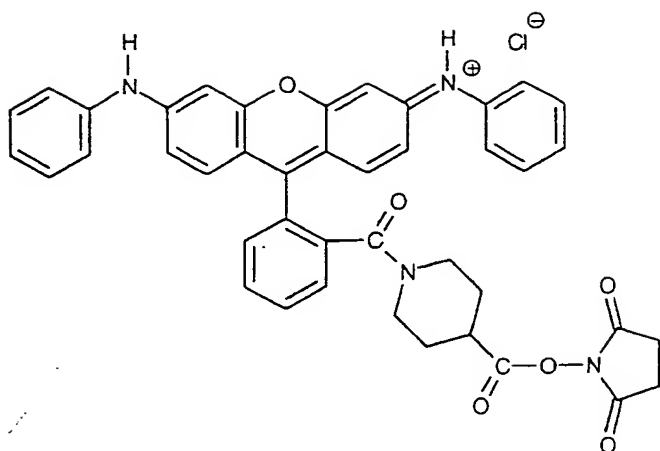
Compound 9

The carboxylic acid 9 (1.5 g) is heated at 70-75 °C in 15 mL of phosphorous oxychloride overnight to generate the acid chloride. Volatiles are removed under reduced pressure. The residue is dissolved in 30 mL of DMF followed by the addition of 2.4 mL of triethylamine and 2.37 g of ethyl  
 5 isonipecotate. After several hours at room temperature, the DMF is removed and the residue is dissolved in 30 mL of chloroform and chromatographed on silica gel to yield 0.65 g of the ethyl ester 10.



Compound 10

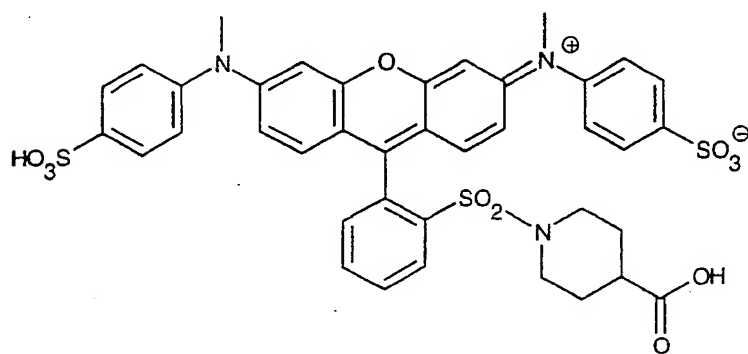
Saponification of the ether ester 10 and conversion to the succinimidyl ester as described in Example 4 yields Compound 11.



Compound 11

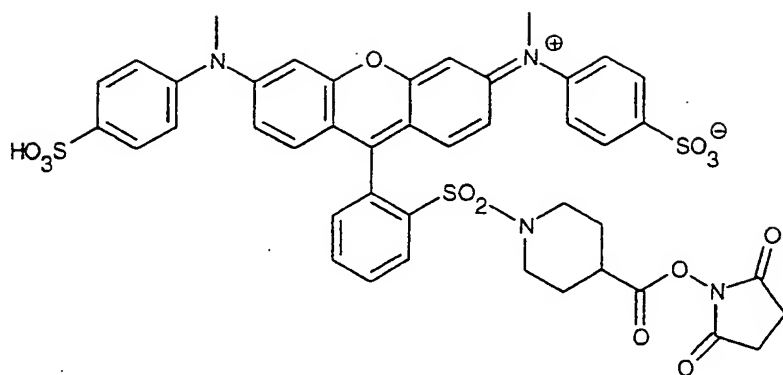
Example 6. Preparation of a succinimidyl ester (13):

To acid 4 (Example 2, 1.5 g) in 20 mL of methylene chloride is added 0.72 mL of chlorosulfonic acid, followed by 4.5 mL of acetic anhydride. The mixture is stirred at room temperature overnight and 100 mL of ether is added precipitate the crude product. Purification is by chromatography on a silica gel column eluting with 9:1 acetonitrile/water to yield 1.0 g of the pure sulfonated derivative 12.



Compound 12

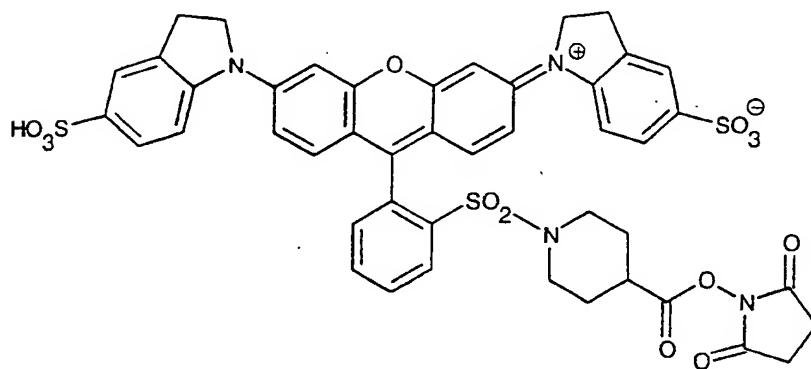
To 0.6 g of acid 12 in 60 mL of DMF at room temperature is added 0.48 mL of diisopropylethylamine and 0.93 g of 2-succinimido-1,1,3,3-tetramethyluronium tetrafluoroborate. The mixture is stirred for 30 minutes. Ethyl acetate (240 mL) is added. The mixture is stirred overnight and the product 13 is recovered by filtration.



Compound 13

Example 7. Preparation of a succinimidyl ester derivative (15):

- 5 Compound 15 is prepared from Compound 6 following the procedure of Example 6,



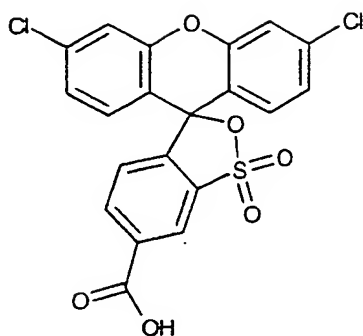
Compound 16

Example 8. Preparation of an acid derivative (18):

10

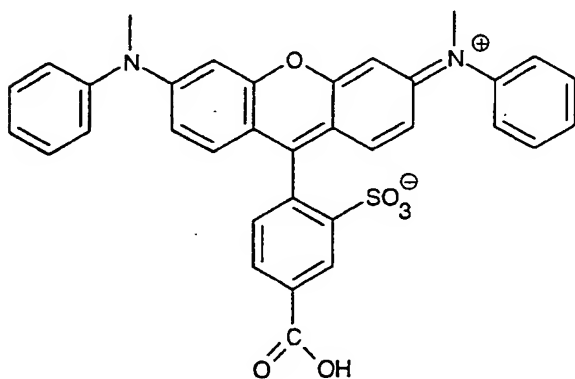
A mixture of 0.83 g of 5-carboxysulfonefluorescein (prepared according to methods provided in International Publication WO 97/39064 (1997)), 1.26 g of phosphorus pentachloride, and 2.5 mL of phosphorus oxychloride is heated at 130 °C for 2 hours. The mixture is cooled and poured onto 80 mL of cold water and stirred for one hour. The solid is filtered and stirred in 20 mL of 5 % NaOH for 30 minutes. The solution is acidified with 20 mL of 6 M HCl. The crude 5-carboxy-3,6-dichlorosulfonefluoranc is filtered and purified by stirring it in 50 mL of methanol for several hours to yield 0.13 g of the dichlorosulfonefluorancarboxylic acid 17.

15



Compound 17

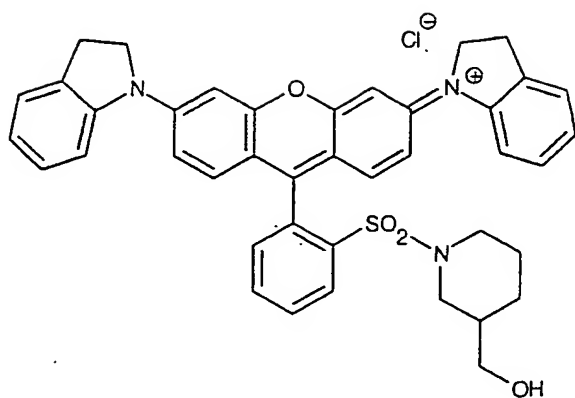
- The fluorene 17 (50 mg) is heated with 1 mL of N-methylaniline at 70 °C for 3 hours. Volatile components are removed at 40 °C *in vacuo*. The residue is dissolved in chloroform and washed with 1 M HCl to yield the crude material, which is purified by recrystallization from chloroform/ethyl acetate to yield 35 mg of the pure acid 18.



Compound 18

10 Example 9. Preparation of an alcohol derivative (20):

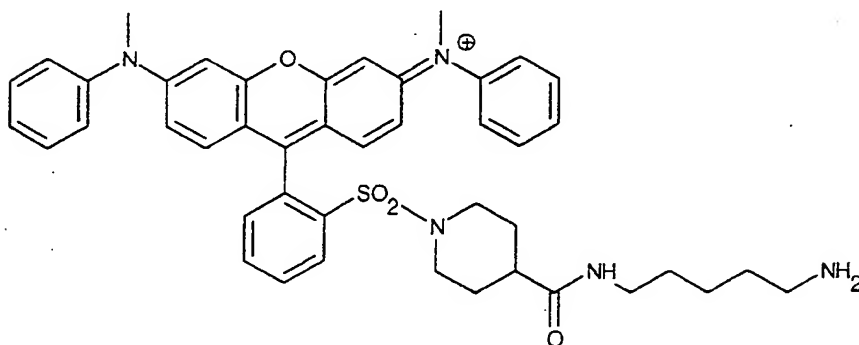
Compound 20 is prepared from Compound C of Example 1 following the procedure of Example 2 via the intermediate sulfonyl chloride derivative, Compound 19.



Compound 20

Example 10. Preparation of a cadaverine derivative (21):

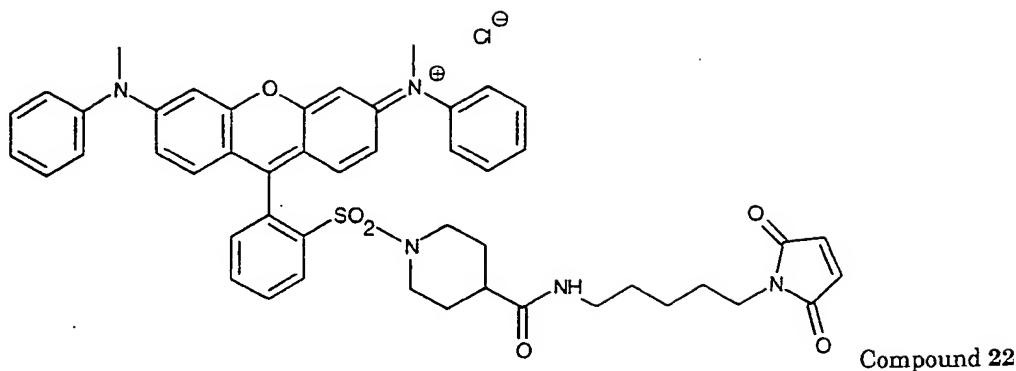
- 5 A mixture of 0.5 g of the succinimidyl ester Compound 5 (Example 3), 0.153 g of mono-N-(t-BOC)-cadaverine and 0.17 mL of diisopropylethylamine in 15 mL of methylene chloride is stirred at room temperature overnight. The reaction mixture is worked up with 1 M HCl and purified on a silica gel column to yield 0.31 g of the pure t-BOC protected sulfonamide. This product is deprotected by dissolving it in 15 mL of methylene chloride and stirring overnight with 0.5 mL of 4
- 10 M HCl in dioxane at room temperature. Following removal of volatiles under reduced pressure, the residue is stirred in 20 mL of ether to yield 0.29 g of the amine 21 as the hydrochloride salt.



Compound 21

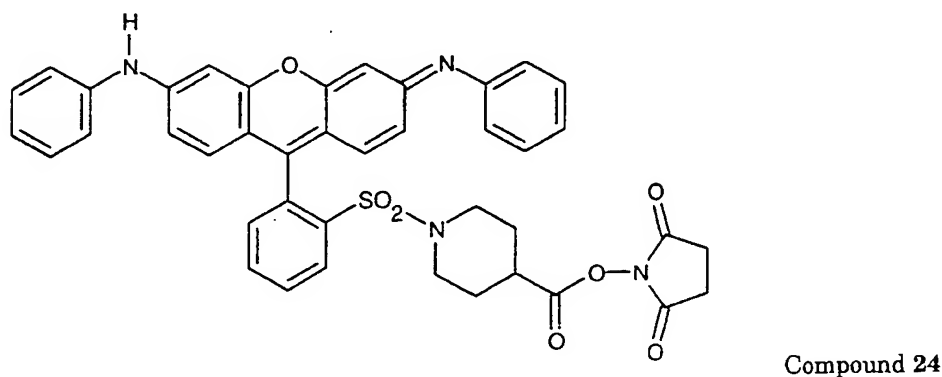
15 Example 11. Preparation of a maleimide derivative (22):

- To the succinimidyl ester Compound 5 of Example 3 (0.5 g) in 15 mL of methylene chloride at room temperature is added 0.23 mL of diisopropylethylamine, followed by 0.26 g of N-(5-aminopentyl)maleimide trifluoroacetic acid salt. The solution is stirred at room temperature for 3
- 20 hours and is then worked up with 1 M HCl. The crude maleimide is purified on a silica gel column to yield 0.35 g of pure product 22.



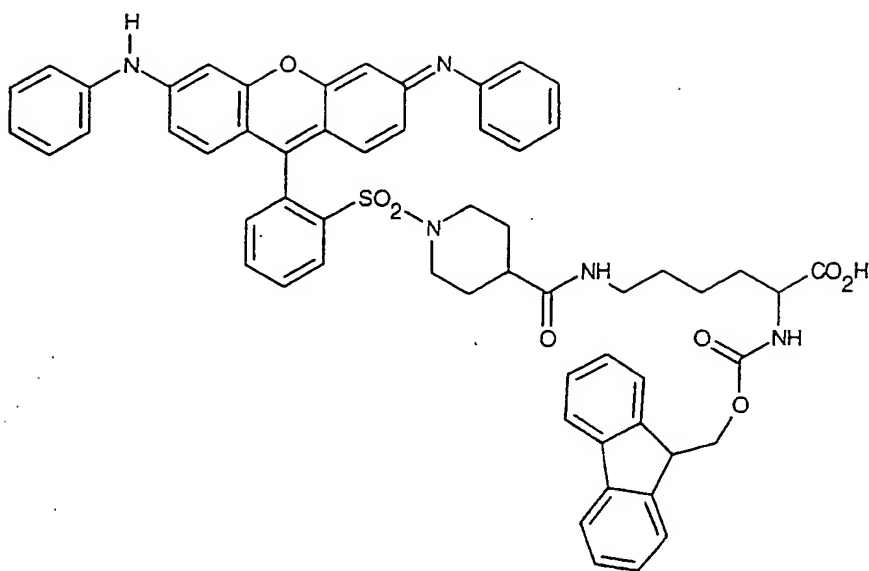
Example 12. Preparation of a succinimidyl ester derivative (24):

Compound A (Example 1) is treated according to the procedure of Example 2 using phosphorous oxychloride and ethyl isonipecotatate, followed by conversion to a succinimidyl ester derivative according to the procedure of Example 4 to yield Compound 24.



Example 13. Preparation of a lysine derivative (25):

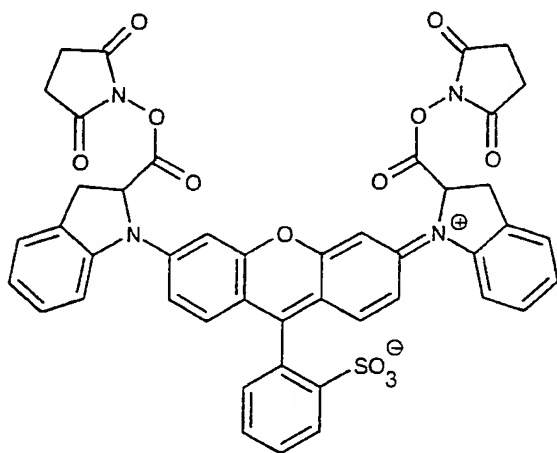
To 0.41 g of Compound 24 in 10 mL of DMF and 3 mL of pyridine at room temperature is added 0.57 g of  $\alpha$ -Fmoc-L-lysine hydrochloride. The mixture is stirred at room temperature for three days, followed by warming at 35 °C for 30 minutes. The solution is poured into 130 mL of cold 1 M HCl. The crude residue is filtered and dried *in vacuo* then purified on a silica gel column to yield 0.55 g of the  $\alpha$ -Fmoc-L-lysine 25.



Compound 25

**Example 14. Preparation of a succinimidyl ester derivative (26):**

- 5 To Compound E (Example 1) is added 2.2 equivalents of ethyldiisopropylamine, followed by 2.4 equivalents of 2-succinimido-1,1,3,3-tetramethyluronium tetrafluoroborate. The reaction mixture is stirred for 20 minutes. Three volumes of ethyl acetate are added to the reaction mixture dropwise, and the resulting solid is collected to obtain the succinimidyl ester 26.



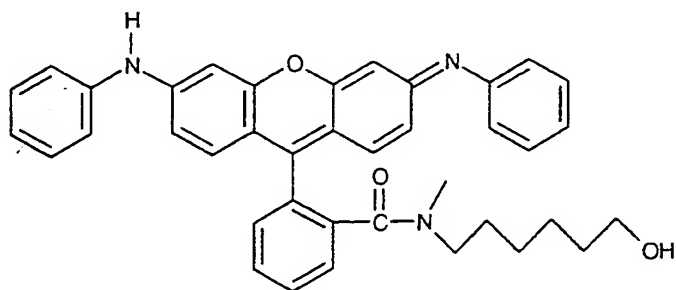
Compound 26

**Example 16. Preparation of a hydroxyl derivative (27):**

- 15 A mixture of 0.75 g of Compound 9 (Example 11) and 7.5 mL of phosphorous oxychloride is heated at 60 °C overnight to generate the corresponding acid chloride. Excess oxychloride is



evaporated and the resulting residue is dried under vacuum at 40 °C for 2 hours. The crude material is then dissolved in a solution of 30 mL of acetonitrile and 3 mL of DMF at room temperature. To this solution is added a mixture of 1.1 g of 6-methylaminohexanol and 1.2 mL of triethylamine in 5 mL of acetonitrile. After the reaction mixture is stirred at room temperature for 2 hours, all volatile components are removed, the resulting residue is partitioned between chloroform and 1 M HCl, and the organic layer is dried and subjected to silica gel chromatography to yield Compound 27.

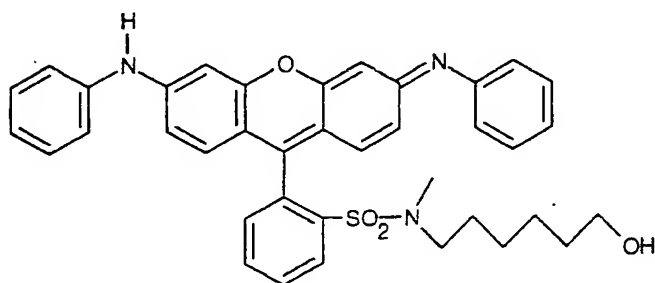


Compound 27

**Example 17. Preparation of a hydroxy derivative (28):**

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Compound 28 is prepared analogously to the method provided for Compound 27 (Example 16), except that Compound A of Example 1 is used instead of Compound 9.



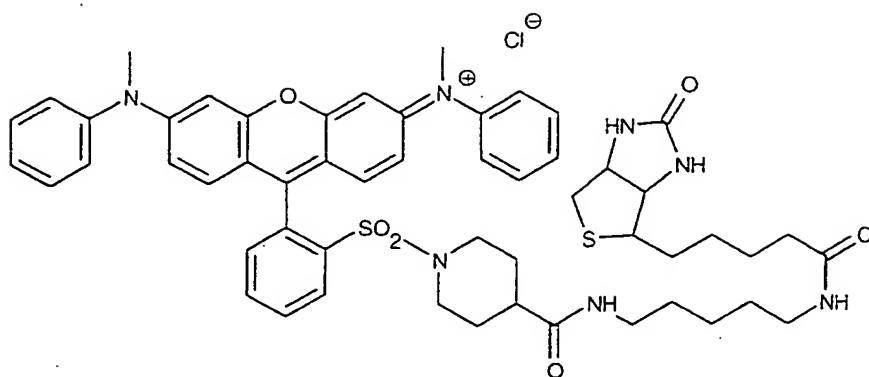
Compound 28

15

**Example 18. Preparation of a biotinylated derivative (29):**

To 25 mg of Compound 5 (Example 3) in 1 mL of DMF is added 17 mg of N-(5-aminopentyl)biotinamide trifluoroacetic acid salt and 6  $\mu$ L of triethylamine. After the reaction mixture is stirred for 2 hours, 6 mL of a 1/1 (v/v) brine and water solution is added dropwise, and the resulting sticky crude material is purified on a silica gel chromatography column to yield Compound 29.

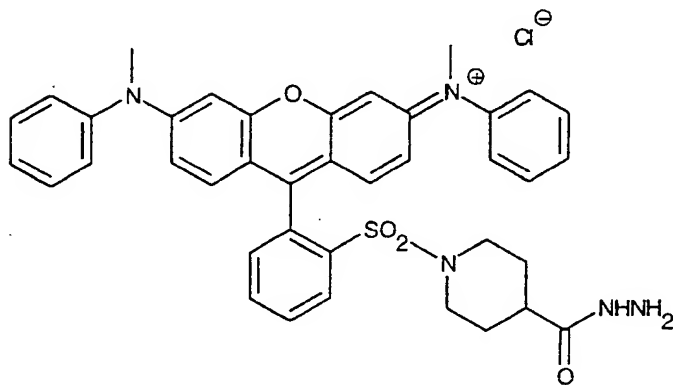
20



Compound 29

Example 19. Preparation of a hydrazine derivative (30):

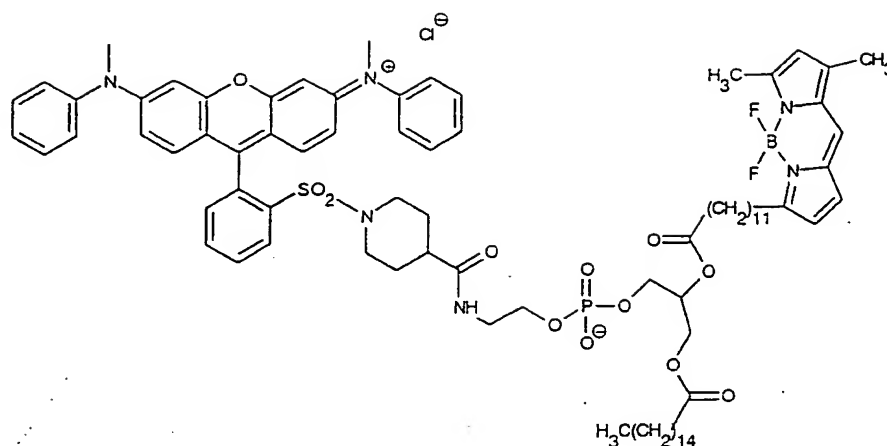
- 5        Compound 5 (Example 3) is treated with a molar excess of hydrazine hydrate in methanol solution. After reaction is complete, the desired product is precipitated by the addition of water, the solid is collected, and purified by silica gel chromatography to yield Compound 30.



Compound 30

10

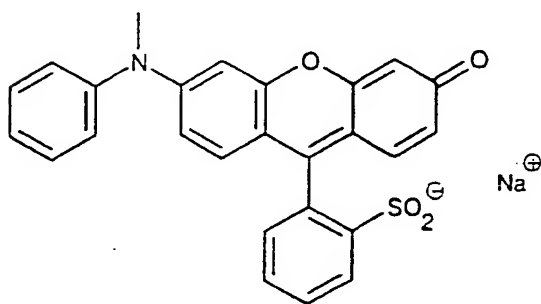
Example 20. Preparation of a phospholipid derivative (31):



Compound 31

To a solution of 2.5 mg (0.003 mmol) of 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoyl)-1-hexadecanoyl-sn-glycero-3-phosphoethanolamine in 500  $\mu$ L of chloroform is added 1.0  $\mu$ L (0.006 mmol) of N,N-diisopropylethylamine followed by 2.5 mg (0.003 mmol) of Compound 5. The mixture is stirred for 16 hours, is then diluted with 15 mL of chloroform, washed with water, and evaporated. The resulting residue is purified by silica gel chromatography to give 2.0 mg of Compound 31 as a purple solid.

Example 21. Preparation of a rhodol quenching compound (32):



Compound 32

To 0.405 g of 3,6-dichlorosulfofluoran in 20 mL of acetonitrile at 0  $^{\circ}$ C is added 0.107 g of N-methylaniline. The mixture is warmed to room temperature and stirred overnight. The volatile components are removed under reduced pressure and 20 mL of dioxane is added, followed by 2 mL of an aqueous 10% solution of NaOH. The reaction mixture is heated at 60  $^{\circ}$ C for 4 hours to generate crude Compound 32, which is isolated by acidification with HCl, followed by chromatographic purification.

Example 22. Evaluation of quenching compound fluorescence:

Quantum yield measurements are made by comparing the integrated fluorescence emission of the quenching compound of the invention with the integrated fluorescence of nile blue (QY = 0.23 in ethanol) at equal dye absorbance, at the excitation wavelength. The fluorescence of buffer alone is subtracted from that of the sample for each measurement. The quenching compounds of the invention are essentially non-fluorescent (Table 4).

Table 4. Spectral properties of selected compounds of the invention.

Quencher	Absorbance maximum (nm)	Emission maximum (nm)	Quantum yield
DABCYL	376	nd	0.0005 <sup>1</sup> 0.0008 <sup>2</sup>
Compound 2	560	604	0.0009
Compound 5	561	608	0.0009
Compound 8	660	760	0.0002
Compound 10	558	609	0.001
Compound 13	560	610	0.002
Compound 16	649	714	0.0001
Compound 27	553	599	0.002
Compound 28	560	635	0.0002
Compound F	505	543	nd
Compound H	548	598	0.009
Compound I	574	570	0.006
Compound J	528	570	nd

<sup>1</sup> relative to fluorescein in TE buffer, pH 9.0, quantum yield 0.92.

<sup>2</sup> relative to quinine sulfate in 0.1 M perchloric acid, quantum yield 0.60.

nd = not determined

**Example 23. Quenching of a fluorescent dye-labeled protein:**

Albumin from bovine serum (BSA), previously labeled with 6.3 moles of fluorescein per mole of protein, is dissolved in 0.1 M bicarbonate buffer (pH 8.3) to give a solution concentration of 5 mg/mL. Three aliquots of 0.4 mL (2 mg) are removed from the resulting solution, and are treated with DMSO solutions of Compound 5 equivalent to 5, 10, and 20 molar equivalents of Compound 5 per mole of BSA, respectively. Each of the resulting solutions are incubated for one hour at room temperature. Hydroxylamine is added (to a final concentration of 0.15 M) to stop the conjugation

reaction. After the samples are incubated for 30 minutes at room temperature, they are subjected to size exclusion chromatography in 0.1 M sodium phosphate buffer (pH 8.0).

The fluorescence emission spectra of the fluorescent starting material and each of the resulting quenched conjugates is recorded by matching the conjugate absorbance at the excitation wavelength (480 nm). The degree of quenching of the conjugates is given in Table 5.

Table 5

quenching compound:BSA ratio	Percent decrease in fluorescein fluorescence
5:1	67.8%
10:1	82.1%
20:1	93.4%

Example 24. Fluorescence quenching of fluorescent avidin and streptavidin via binding of quenching compound-labeled biotin:

A variety of fluorescent dye-labeled avidin or streptavidin conjugates (Molecular Probes, Inc.) are treated with Compound 29, and the effect of binding the quenching compound labeled biotin on fluorescence is measured.

General procedure: Samples of each fluorescently labeled avidin or streptavidin conjugate (0.5 mg each) are dissolved in 0.1 M sodium phosphate (pH 7.5). To establish normal fluorescence, 0.5 mg of each conjugate is treated with non-labeled biotin dissolved in DMSO to obtain a final molar ratio of 10 moles of biotin per mole of protein. To evaluate fluorescence quenching, 0.5 mg of each conjugate is treated with Compound 29 at a final molar ratio of 10 moles of biotin conjugate per mole of protein. Each sample is incubated for one hour at room temperature, then purified using size exclusion chromatography in 0.1 M sodium phosphate (pH 7.5, or pH 8.0 in the case of avidin-fluorescein).

The fluorescence emission spectra of the quenched and control samples, matched for absorbance at the excitation wavelength, show that binding Compound 29 produces a decrease in the fluorescence, relative to the biotin control, in all cases.

Table 6. Percent Quenching upon Binding Compound 29:

Protein-Dye Conjugate associated with Compound 29	Dyes per Protein	$\lambda_{Ex}$ (nm)	Relative QY*	Percent Quenching
Avidin-fluorescein	3.4	480	0.014	98.6
Streptavidin-MARINA BLUE™	5.2	350	0.072	92.8
Streptavidin-ALEXA™ 350	4.3	350	0.026	97.4

Streptavidin-TEXAS RED®X	3.1	560	0.003	99.7
Streptavidin-RPE†	n.a.	495	0.151	84.9
Streptavidin-APC‡	n.a.	615	0.576	42.4

\* Relative quantum yield is the quantum yield of the quenched protein-dye conjugate relative to the quantum yield of the free, unconjugated, fluorophore.

† RPE = R-phycoerythrin

‡ APC = allophycocyanin

5

#### Example 25. Preparation of oligonucleotide conjugates of quenching compounds:

Eighteen-base oligonucleotide conjugates of quencher dyes are prepared using standard methods. Typically, a C-6 TFA primary amine is synthetically incorporated on the 5' end of the oligonucleotide of interest as a phosphoramidite, and is subsequently reacted with a succinimidyl ester derivative of a quenching compound of the invention.

The succinimidyl ester derivative is dissolved in DMSO at a concentration of about 12.5 mg/mL. The amine-modified oligonucleotide is dissolved in water at a concentration of 25 mg/mL. A fresh solution of 0.1 M sodium borate, pH 8.5 is prepared. In a microfuge tube, 4 µL of the oligonucleotide solution is combined with 200 µg of the quenching compound solution and 100 µL sodium borate buffer. Samples are incubated 4 hours to overnight at room temperature, and the nucleic acids are precipitated by addition of 1/10<sup>th</sup> volume 0.3 M NaCl and 2.5 volumes cold absolute ethanol. Samples are incubated for 30 minutes at -20 °C and centrifuged at 15,000 x g in a microfuge for 30 minutes. The supernatant fluid is decanted and the pellet dried under vacuum.

Alternatively, the oligonucleotide conjugate is prepared by reaction of a maleimide derivative of a quenching compound of the invention with an oligonucleotide that has been derivatized by a thiol that has been incorporated via a phosphoramidite.

Conjugates are further purified by reverse phase HPLC, using a C8 reverse phase column and a gradient of 5–95% acetonitrile in 0.1 M TEAA, pH 7. Absorbance and fluorescence emission spectra are determined in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5. Quantum yield measurements are made as described above (Example 22). Oligonucleotide conjugates of the quenchers are essentially nonfluorescent (Table 7).

Table 7. Spectral properties of amine-oligonucleotide conjugates of the quencher dyes.

Compound	Excitation wavelength (nm)	QY of the conjugate	Fluorescence increase relative to free dye
Compound 5	560	0.003	3.5-fold
Compound 8	630	0.0006	3-fold
Compound 13	560	0.004	2-fold
Compound 16	630	0.0008	4–8-fold

30

The absorbance and emission maxima for the conjugates are shifted typically 5–15 nm relative to those of the free dye.

Example 26. Preparation of an oligonucleotide conjugate, substituted with both a fluorophore and a quenching compound:

Oligonucleotides conjugated to a fluorescein fluorophore at one terminus and a quenching compound of the invention at the other terminus are prepared using a 3' or 5' fluorescein phosphoramidite and an amine modifier at the other terminus of the oligonucleotide, followed by labeling with a succinimidyl ester derivative of the invention (as described above in Example 25), or by synthesis of oligonucleotides containing an amino modifier on one terminus and a thiol at the other terminus, followed by sequential reaction with a maleimide derivative and succinimidyl ester derivative of the fluorophore and quenching compound, or vice versa. The fluorescence of the resulting conjugates is measured at equal conjugate concentration. Selected compounds of the instant invention quench the fluorescence of fluorescein much more efficiently than does DABCYL and all of the quenchers quench tetramethylrhodamine much more efficiently than does DABCYL (as shown in Table 8).

Table 8. The fluorescence of selected fluorophore-labeled and quenching compound-labeled eighteen-base oligonucleotides.

Quenching compound	Fluorescence emission relative to unquenched fluorescein conjugate	Fluorescence emission relative to unquenched tetramethylrhodamine conjugate
None	100%	100%
DABCYL	14%	92%
Compound 5	4.5%	6%
Compound 8	12%	3%
Compound 13	12%	--
Compound 16	4.5%	4%

Example 27. Preparation of doubly labeled oligonucleotide conjugates:

Random-sequence oligonucleotides 10, 20, 30 and 40 bases in length are labeled with fluorescein at their 3' terminus using a fluorescein phosphoramidite, and with Compound 5 or DABCYL at their 5' terminus, by reacting the amine-modified oligonucleotide with the succinimidyl ester derivative (as described above, Example 2). Resulting conjugates are purified by reverse phase HPLC. The fluorescence of equal amounts of these doubly labeled conjugates is determined relative to the fluorescence of fluorescein conjugates lacking the quencher dye. Compound 5 quenches the

fluorescein conjugates more efficiently than DABCYL does, until the conjugates reach about 30 bases in length.

Table 9. Relative % fluorescence intensity of the quencher conjugates compared to unquenched fluorescein conjugates of the same length.

Oligonucleotide length (in bases)	DABCYL	Compound 5
10	17	2.3
20	49	18
30	62	46
40	58	62

Example 28. Hybridization of doubly labeled oligonucleotide conjugates to unlabeled complementary oligonucleotides:

Solutions are prepared containing 1  $\mu\text{g/mL}$  18-base oligonucleotide conjugates of a quenching compound of the invention attached to the 5' terminus, as well as a fluorescein or tetramethylrhodamine fluorophore on the 3' terminus. The oligonucleotide conjugates are hybridized with 40  $\mu\text{g/mL}$  reverse complement oligonucleotide in TE buffer at pH 9.0. The samples are heated for 10 minutes at 65  $^{\circ}\text{C}$ , allowed to cool slowly to room temperature, and are then incubated at room temperature for 60 minutes, protected from light. A portion of each sample is transferred to a microplate well and the fluorescence emission of the sample is determined at 530 $\pm$ 9 nm (with 480 $\pm$ 9 nm excitation) for fluorescein and at 580 $\pm$ 9 nm (with 535 $\pm$ 9 nm excitation) for tetramethylrhodamine. In each case, the fluorescence is compared to the fluorescence of a buffer solution alone.

All of the conjugates of the invention exhibit an increase in fluorescence upon hybridization, even oligonucleotides labeled with fluorescein or tetramethylrhodamine alone (Table 10).

Table 10. Effect of hybridization on fluorescence quenching.

Quenching Compound	% Fluorescence Recovered upon Hybridization*	
	Fluorescein	Tetramethylrhodamine
None	113%	230%
DABCYL	33%	140%
Compound 5	31%	111%
Compound 8	35%	49%
Compound 13	35%	nd
Compound 16	34%	27%

\* The fluorescence exhibited by the double-labeled oligonucleotide upon hybridization, divided by the fluorescence exhibited by an oligonucleotide labeled with the fluorophore alone.



Because the quenched oligonucleotides initially exhibited extremely low fluorescence, they show larger increases upon hybridization, and therefore the conjugates that are the most efficiently quenched prior to hybridization exhibit the largest increase in fluorescence. This property is utilized to formulate a homogenous assay method to detect the presence of specific complementary DNA sequences in a sample. Several of the compounds of the invention quench fluorescence more efficiently than DABCYL in this application. Similarly, doubly labeled oligonucleotides that form structures that enhance quenching, such as hairpin or stem loop structures, as in BEACON probes, can also be used in this application.

Table 11. Fluorescence enhancement upon hybridization.

Quenching Compound	Fluorescence Enhancement*	
	Fluorescein label	Tetramethylrhodamine label
None	1.1	2.3
DABCYL	2.4	1.4
Compound 5	6.3	19
Compound 8	2.9	7
Compound 13	2.8	nd
Compound 16	8.2	19

\* The observed increase in fluorescence of the double-labeled oligonucleotide upon hybridization.

Example 29. Hybridizing oligonucleotide conjugates of quenching compounds with fluorophore labeled oligonucleotides:

Oligonucleotides conjugated to a quenching compound at one terminus quench the fluorescence of fluorophore labeled nucleotides upon hybridization. Labeled oligonucleotides are prepared as described above (Examples 25 and 26), and hybridized with their reverse complements. Samples containing 2  $\mu\text{g/mL}$  quenching compound-labeled 18 base oligonucleotides and 200  $\text{ng/mL}$  fluorescein-labeled reverse complement oligonucleotides in 10 mM Tris-HCl, 1 mM EDTA, pH 9.0, are hybridized and their fluorescence is determined as described above (Example 22). The quenching compound oligonucleotides efficiently quench the fluorescence of fluorescein that is localized at the same end of hybridized oligonucleotides, but quench the fluorescence of distant fluorophores more poorly.

Table 12. Effect of fluorophore quenching compound proximity on quenching efficiency.

<u>Quencher</u>	Relative Fluorescence of the hybrid (%) <sup>1</sup>	
	3'-end label <sup>2</sup>	5'-end label <sup>2</sup>
None	79	75
DABCYL	14	98
Compound 5	6.6	68
Compound 8	11	89
Compound 13	10	82
Compound 16	13	84

1 Relative fluorescence is the fluorescence of the hybrid divided by that obtained for the fluorophore-labeled oligonucleotide alone.

2 The position of the end label indicates the position of the fluorophore label on the reverse complement oligonucleotide. The quenching compound is attached at the 5' terminus of the oligonucleotide.

Example 30. Quenching fluorescence of nucleotides added enzymatically to the 3' end of a primer:

10 An eighteen-base oligonucleotide is labeled with Compound 5 on its 5' terminus, as described above (Example 25). The resulting conjugate is incubated with terminal deoxynucleotidyl transferase under standard assay conditions for 3' end elongation, in the presence of fluorophore-labeled dUTP conjugates, as follows: The oligonucleotide conjugate (650 ng) is incubated with 1  $\mu$ L of 25 mM fluorophore-labeled nucleotide, 0.5 mM CoCl<sub>2</sub>, and 0.2 M potassium cacodylate, 25 mM

15 Tris-HCl, pH 6.6, 2 mM DTT, and 250  $\mu$ g/mL bovine serum albumin for 60 minutes at 37 °C. A one-fifth volume of a solution containing 50% glycerol and 0.01% bromophenol blue is added to each reaction, and the samples are separated by electrophoresis on a 20% polyacrylamide/8 M urea minigel in TBE buffer (45 mM Tris-borate, 1 mM EDTA), under conditions that resolve single nucleotide additions to the oligonucleotide. Samples containing oligonucleotides that are lacking the

20 quenching compound are processed in parallel, for use as size standards. Gels are visualized using a 300-nm UV transilluminator combined with Polaroid black and white photography, or using a laser scanner. The gels are post-stained with a fluorescent nucleic acid stain, such as SYBR® Gold stain, and band fluorescence is visualized in the same way. The size of the oligonucleotides is determined based on comparisons of electrophoretic migration with the unlabeled standard. Quenching is

25 detected as lack of fluorescence or visibility of a band of a particular size from the pattern visible in the standard. Where the fluorophore is fluorescein or Texas Red® dye, the label fluorescence is readily quenched by the 5'-bound quenching compound. The fluorescence of Cascade Blue® dye is not as efficiently quenched. As additional nucleotides are added, the ones most distant from the quenching compound begin to exhibit fluorescence.

This technique is useful as a gel-based method for quantitating terminal transferase activity. Enzyme activity in an unknown sample is determined by comparison of the number of added nucleotides per template or the number of templates with added nucleotides of a certain length with the numbers obtained using a standard amount of enzyme activity following a standard reaction time interval.

Example 31. Quenching of a fluorescent oligonucleotide by enzymatic incorporation of a quenching compound conjugate of nucleotide triphosphate via primer extension:

A short oligonucleotide, having 6 to about 20 bases, is labeled with a fluorophore such as fluorescein, tetramethylrhodamine or Texas Red® dye, on its 5' terminus, and then purified via HPLC using standard methods (as described above, Examples 25 and 26). For template-driven reactions, the oligonucleotide is hybridized to an appropriate template, and incubated with a quenching compound-labeled nucleotide or deoxynucleotide in an appropriate buffered solution, in the presence of samples thought to contain an appropriate DNA or RNA polymerase. Enzyme activity is determined by measuring the rate of fluorescence loss from the solution, versus the rate of loss observed from solutions containing known amounts of enzyme activity. Terminal deoxynucleotidyltransferase activity is assayed by determining the rate of fluorescence loss from the solution upon incubation with samples thought to contain terminal deoxynucleotidyltransferase activity. For measurement of terminal deoxynucleotidyl transferase activity, fluorophore-labeled templates are incubated with quenching compound-labeled nucleotides in the buffer described above (Example 33) for a set time interval, and fluorescence is measured in a fluorescence microplate reader or fluorometer.

To measure reverse transcriptase activity, 2 µg mRNA is combined with 5 µg fluorophore labeled poly dT(16) oligomer in 10 mM Tris-HCl, pH. 8.0, 1 mM EDTA; the mixture is heated to 70 °C for 10 minutes and then chilled on ice. A solution containing 2 µL reverse transcriptase (200 units/µL for the standard, or unknown amounts), 500 µM dATP, 500 µM dCTP, 500 µM dGTP, 200 µM dTTP, and 60 µM quenching compound-labeled dUTP is prepared and added to the RNA. The reaction is allowed to proceed for 2 hours at 42 °C. The fluorescence of the solution is measured in a fluorescence microplate reader or fluorometer versus a standard. The decrease in fluorescence in comparison to samples lacking enzyme activity is directly related to the activity of the enzyme in the reaction.

To measure Klenow DNA polymerase activity, 1 µg random sequence 9-mer oligonucleotides labeled with a fluorescent dye are combined with 2.5 µg genomic DNA in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. The mixture is boiled for 2 minutes and chilled on ice. A reaction mixture containing 25 µM dATP, 25 µM dCTP, 25 µM dGTP and 10 µM dTTP, plus 40 µM quenching compound-labeled dUTP in 1 mM Tris-HCl, pH 7.5, 5 mM NaCl, 0.01 mM EDTA, pH 8.0, 5 mM dithiothreitol is combined with samples thought to contain DNA polymerase. The reaction mixture is combined with

the DNA mixture and incubated at 37 °C for 2 hours. The fluorescence of the sample is measured versus standards, as described above.

Example 32. Using quenching compounds to measure nuclease activity:

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Oligonucleotide conjugates labeled with both a quenching compound at one terminus and a fluorophore at the other terminus are prepared as described above (Examples 25 and 26). For measuring single-stranded nuclease activity, the conjugates are incubated in the presence of samples thought to contain nuclease activity in the presence of an appropriate buffer and the resulting fluorescence increase in the sample is compared to that obtained using standards of known nuclease concentration. To measure double-stranded nuclease activity, double-stranded templates are prepared by hybridizing two oligonucleotides to one another, or by chemically modifying a double-stranded template using reagents such as platinum complexes of fluorophores and quenchers (as described above), or by using an enzyme such as a terminal transferase to add nucleotides to the end of a template (as described above in Examples 30 and 31). Samples thought to contain nuclease activity are incubated with such templates in the presence of appropriate buffers and the increase in fluorescence compared to a standard, as described above.

20

Example 33. Using quenching compounds to measure ligase activity:

Oligonucleotide hexamers labeled at the 5' terminus with a quenching compound are prepared as described above. Oligonucleotide hexamers labeled with a fluorophore at the 3' terminus and phosphate at the 5' terminus are prepared as described above excepting that the phosphate is alternatively applied by standard methods using a phosphoramidite or by enzymatic means, such as T4 polynucleotide kinase.

A reaction mixture is prepared that contains about 5 µg of each oligonucleotide conjugate, 0.5 mM ATP, and samples thought to contain ligase activity, in 1 mM MgCl<sub>2</sub>, 2 mM dithiothreitol, 5 µg/mL bovine serum albumin, and 5 mM Tris-HCl, pH 7.7, in a volume of 20 µL. The reaction mixtures are incubated for 2 hours to overnight at 22° C, and the sample fluorescence is measured. As the quenching compound-labeled oligonucleotides do not contain a free 5' phosphate, they cannot ligate to one another, and as the fluorophore-labeled oligonucleotides do not contain a free 3' hydroxyl, they cannot ligate to one another. Thus the only products of ligation will be a dimer of the two oligonucleotides and the fluorescence decrease observed during the course of the reaction is a measure of ligase activity. Alternatively, RNA oligonucleotides are used as templates to measure RNA ligase activity or splicing activity.

Example 34. Labeling large DNA molecules with platinum quenching compound complexes:

A quenching compound platinum complex is synthesized by adapting the methods provided in U.S. Patent No. 5,714,327 to Houthoff et. al. (1998) and a 1 mg/mL solution of the quenching compound complex is prepared in water. This solution is then diluted into water, and 10 ng to 3 µg of the quenching compound complex is added to a microfuge tube containing 500 ng of plasmid DNA. The volume is brought up to 25 µL with water, and the samples heated at 65 °C for 15 minutes. Five µL of 1% diethyldithiocarbamic acid sodium salt solution is added to stop the reaction, samples are mixed and cooled to room temperature, and 10 µL of each reaction is loaded onto a 1% agarose minigel. The gel is electrophoresed in 0.5X TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8), stained with SYBR® Gold stain, and photographed with 300 nm transillumination, through a SYBR® Gold photographic filter. Samples labeled with 10 ng to 0.3 µg of the quenching compound complex are very fluorescent. Samples labeled with 0.5 µg to 1 µg of the quenching compound complex show only weak fluorescence. Samples labeled with 1.5 µg to 3 µg of the quenching compound complex are essentially non-fluorescent. Similarly, samples labeled with sufficient amounts of the quenching compound complex are able to quench the fluorescence of bound hybridization probes that are themselves labeled with reactive fluorophore labels or fluorescent nucleotides.

Example 35. Preparation of quenched double-stranded DNA:

Oligonucleotides are prepared that are either labeled with a fluorophore at a strand terminus, or within the oligonucleotide sequence itself, using standard methods as described above. The oligonucleotides are then used as primers for PCR or are otherwise enzymatically extended using standard methods. A quenching compound platinum complex is prepared (as described in U.S. Patent No. 5,714,327 to Houthoff et. al. (1998)) and dissolved in water at a final concentration of 1 mg/mL. DNA (500 ng) is combined with 1.5 µg of the quenching compound platinum complex and incubated in a total volume of 25 µL water for 15 minutes at 65 °C. The reaction is chilled in an ice bath to stop it. The quenched DNA is not visible after gel electrophoresis, even when stained with a fluorescent nucleic acid stain or incubation in solution with a fluorescent nucleic acid stain.

Example 36. Using quenching compounds to assay topoisomerase activity:

Quenched DNA is prepared as described above, using a circular single stranded DNA template, such as an M13 or ϕX174 phage DNA genome, and a quenching compound platinum complex (Example 34). A fluorophore-labeled oligonucleotide is then hybridized to the quenched DNA. Samples thought to contain topoisomerase activity are combined with the template under optimal reaction conditions for the enzyme, and the reaction is allowed to proceed for an appropriate period of time. Enzyme activity is measured as fluorescence increase for the solution, using a fluorescence microplate reader or fluorometer.

Example 37. Detection of phospholipase activity:

5 A 10  $\mu$ M solution of the phospholipase substrate Compound 31 in 100 mM Tris-HCl, pH 8.0, 0.5 mM  $\text{CaCl}_2$  is prepared. Aliquots of the solution are then incubated at 37 °C with varying concentrations of the calcium-dependent phospholipase enzyme  $\text{PLA}_2$  for up to 120 minutes. Upon incubation, the solution becomes highly fluorescent. In the absence of  $\text{PLA}_2$ , however, the sample solution remains essentially nonfluorescent.

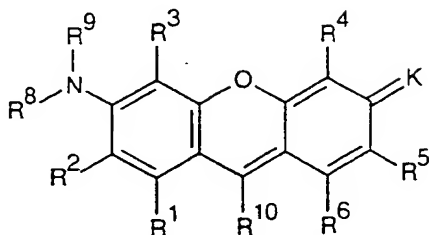
10 The enzymatic activity of  $\text{PLA}_2$  is detected by measuring the fluorescence intensities of the reaction mixtures using a fluorescence plate reader with the standard filter set for fluorescein. Fluorescence intensity correlates very well with the enzyme activity of  $\text{PLA}_2$  (correlation coefficient of 0.9995, see Figure 1). However, there is no change of fluorescence when the substrate is incubated with  $\text{PLA}_2$  in the buffer without 0.5 mM  $\text{CaCl}_2$ , or in the presence of 2 mM EGTA, indicating that this  $\text{PLA}_2$  (Sigma P-6534) is highly calcium-dependent.

15

It is to be understood that, while the foregoing invention has been described in detail by way of illustration and example, numerous modifications, substitutions, and alterations are possible without departing from the spirit and scope of the invention as described in the following claims.

What is claimed is:

1. A compound, having the formula



wherein

R<sup>1</sup> and R<sup>6</sup> are H;

R<sup>2</sup>, R<sup>3</sup>, R<sup>4</sup> and R<sup>5</sup> are independently H, F, Cl, Br, I, CN; or C<sub>1</sub>-C<sub>18</sub> alkyl, or C<sub>1</sub>-C<sub>18</sub> alkoxy, where each alkyl or alkoxy is optionally further substituted by F, Cl, Br, I, a carboxylic acid, a salt of carboxylic acid, or a carboxylic acid ester of a C<sub>1</sub>-C<sub>6</sub> alcohol; or -SO<sub>3</sub>X where X is H or a counterion;

or R<sup>1</sup> taken in combination with R<sup>2</sup>, or R<sup>6</sup> taken in combination with R<sup>5</sup> is a fused six-membered aromatic ring;

R<sup>8</sup> and R<sup>9</sup> are independently H, C<sub>1</sub>-C<sub>6</sub> alkyl, C<sub>1</sub>-C<sub>6</sub> carboxyalkyl, C<sub>1</sub>-C<sub>6</sub> sulfoalkyl, a salt of C<sub>1</sub>-C<sub>6</sub> carboxyalkyl, or a salt of C<sub>1</sub>-C<sub>6</sub> sulfoalkyl, wherein the alkyl portions are optionally substituted by amino, hydroxy, carboxylic acid, a salt of carboxylic acid, or a carboxylic acid ester of a C<sub>1</sub>-C<sub>6</sub> alkyl; or -L-R<sub>x</sub>; or -L-S<sub>c</sub>; or one or more of R<sup>8</sup> and R<sup>9</sup> is a Q moiety; or R<sup>8</sup> taken in combination with R<sup>9</sup> forms a saturated 5- or 6-membered heterocycle that is optionally fused to a Q moiety and optionally further substituted by methyl, carboxylic acid, a salt of carboxylic acid, a carboxylic acid ester of a C<sub>1</sub>-C<sub>6</sub> alkyl, or -L-R<sub>x</sub>, or -L-S<sub>c</sub>;

or R<sup>8</sup> in combination with R<sup>2</sup>, or R<sup>9</sup> in combination with R<sup>3</sup>, or both, form a 5- or 6-membered ring that is saturated or unsaturated, and is optionally substituted by one or more C<sub>1</sub>-C<sub>6</sub> alkyls or -CH<sub>2</sub>SO<sub>3</sub>X;

wherein each Q moiety is 1-4 fused aromatic or heteroaromatic rings that is optionally substituted by halogen, cyano, sulfo, alkali or ammonium salt of sulfo, carboxy, alkali or ammonium salt of carboxy, nitro, alkyl, perfluoroalkyl, alkoxy, alkylthio, amino, monoalkylamino, dialkylamino; alkylamido; or

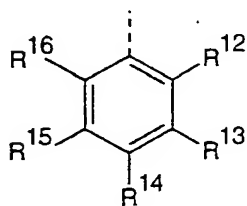
is substituted by  $-L-R_x$ ; or is substituted by  $-L-Sc$ ; wherein each heteroaromatic ring in  $Q$  is a 5- or 6-membered aromatic heterocycle having 1 to 3 heteroatoms selected from the group consisting of O, N or S in any combination; and

5 K is O or  $N \cdot R^{18}R^{19}$ ;

wherein  $R^{18}$  and  $R^{19}$  are independently H,  $C_1-C_6$  alkyl,  $C_1-C_6$  carboxyalkyl,  $C_1-C_6$  sulfoalkyl, a salt of  $C_1-C_6$  carboxyalkyl, or a salt of  $C_1-C_6$  sulfoalkyl, wherein the alkyl portions are optionally substituted by amino, hydroxy, carboxylic acid, a salt of carboxylic acid, or a carboxylic acid ester of a  $C_1-C_6$  alkyl; or  $-L-R_x$ ; or  $-L-Sc$ ; or one or more of  $R^{18}$  and  $R^{19}$  is a  $Q$  moiety; or  $R^{18}$  taken in combination with  $R^{19}$  forms a saturated 5- or 6-membered heterocycle that is a piperidine, or a pyrrolidine that is optionally fused to a  $Q$  moiety, and optionally further substituted by methyl, carboxylic acid, a salt of carboxylic acid, a carboxylic acid ester of a  $C_1-C_6$  alkyl; or  $-L-R_x$ ; or  $-L-Sc$ ;

15 or  $R^{18}$  in combination with  $R^4$ , or  $R^{19}$  in combination with  $R^5$ , or both, form a 5- or 6-membered ring that is saturated or unsaturated, and is optionally substituted by one or more  $C_1-C_6$  alkyls or  $-CH_2SO_3X$  moieties;

$R^{10}$  is H, CN, a carboxylic acid, a salt of carboxylic acid, or a carboxylic acid ester of a  $C_1-C_6$  alcohol; or  $R^{10}$  is a saturated or unsaturated  $C_1-C_{18}$  alkyl that is optionally substituted one or more times by F, Cl, Br, carboxylic acid, a salt of carboxylic acid, a carboxylic acid ester of a  $C_1-C_6$  alcohol,  $-SO_3X$ , amino, alkylamino, or dialkylamino, the alkyl groups of which have 1-6 carbons; or  $R^{10}$  has the formula



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where  $R^{12}$ ,  $R^{13}$ ,  $R^{14}$ ,  $R^{15}$  and  $R^{16}$  are independently H, F, Cl, Br, I,  $-SO_3X$ , a carboxylic acid, a salt of carboxylic acid, CN, hydroxy, amino, hydrazino; or  $C_1-C_{18}$  alkyl,  $C_1-C_{18}$  alkoxy,  $C_1-C_{18}$  alkylthio,  $C_1-C_{18}$  alkanoylamino,  $C_1-C_{18}$  alkylaminocarbonyl,  $C_2-C_{36}$  dialkylaminocarbonyl,  $C_1-C_{18}$  alkyloxycarbonyl, or  $C_6-C_{18}$  arylcarboxamido, the alkyl or aryl portions of which are optionally substituted one or more times by F, Cl, Br, I, hydroxy, carboxylic acid, a salt of carboxylic acid, a carboxylic acid ester of a  $C_1-C_6$  alcohol,  $-SO_3X$ , amino, alkylamino, dialkylamino or alkoxy, the alkyl portions of each having 1-6 carbons; or one pair of adjacent substituents  $R^{13}$  and  $R^{14}$ ,  $R^{14}$  and  $R^{15}$  or



R<sup>15</sup> and R<sup>16</sup>, when taken in combination, form a fused 6-membered aromatic ring that is optionally further substituted by carboxylic acid, or a salt of carboxylic acid; or one of R<sup>12</sup>, R<sup>13</sup>, R<sup>14</sup>, R<sup>15</sup> and R<sup>16</sup> is -L-R<sub>x</sub> or -L-Sc;

5 provided that at least one of R<sup>8</sup>, R<sup>9</sup>, R<sup>18</sup>, and R<sup>19</sup> is, or is fused to, a Q moiety; and

further provided that at least one of R<sup>8</sup>, R<sup>9</sup>, R<sup>12</sup>, R<sup>13</sup>, R<sup>14</sup>, R<sup>15</sup>, R<sup>16</sup>, R<sup>18</sup>, or R<sup>19</sup> is -L-R<sub>x</sub> or -L-Sc; or at least one Q moiety is substituted by -L-R<sub>x</sub> or -L-Sc; wherein

10 L is a covalent linkage; and

R<sub>x</sub> is a reactive functional group that is a maleimide, isocyanate, isothiocyanate, a phosphoramidite, a reactive platinum complex, perfluorobenzamido, azidoperfluorobenzamido, a succinimidyl ester, a sulfosuccinimidyl ester, an alkali or alkaline earth metal salt of a sulfosuccinimidyl ester, a  
 15 symmetric anhydride, a mixed anhydride of a chloroformate having 2-8 carbons, a mixed anhydride of a carboxylic acid or perfluorinated carboxylic acid having 2-8 carbons, a mixed anhydride of a sulfonic acid or fluorinated sulfonic acid having 1-8 carbons, or an ester of a phenol or a naphthol that is further substituted one or more times by nitro, sulfo, carboxy, alkali or alkaline earth metal salt of sulfo or carboxy, cyano, fluoro, chloro, or trifluoromethyl; or R<sub>x</sub> is the adduct of a carboxylic  
 20 acid and a carbodiimide having 2-14 carbons; and

Sc is a conjugated substance.

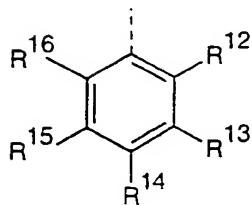
2. A compound, as claimed in Claim 1, wherein at least one of R<sup>8</sup>, R<sup>9</sup>, R<sup>12</sup>, R<sup>13</sup>, R<sup>14</sup>, R<sup>15</sup>, R<sup>16</sup>, R<sup>18</sup>, or  
 25 R<sup>19</sup> is -L-R<sub>x</sub>; or at least one Q moiety is substituted by -L-R<sub>x</sub>; and R<sub>x</sub> is a phosphoramidite, a reactive platinum complex, a succinimidyl ester of a carboxylic acid, a haloacetamide, a hydrazine, an isothiocyanate, a maleimide group or an azidoperfluorobenzamido group.

3. A compound, as claimed in Claim 2, wherein R<sub>x</sub> is a phosphoramidite, a reactive platinum  
 30 complex, or a succinimidyl ester of a carboxylic acid.

4. A compound, as claimed in Claim 1, wherein at least one of R<sup>8</sup>, R<sup>9</sup>, R<sup>12</sup>, R<sup>13</sup>, R<sup>14</sup>, R<sup>15</sup>, R<sup>16</sup>, R<sup>18</sup>, or  
 R<sup>19</sup> is -L-Sc; or at least one Q moiety is substituted by -L-Sc; and Sc is an antigen, a steroid, a  
 vitamin, a drug, a hapten, a metabolite, a toxin, an environmental pollutant, an amino acid, a  
 35 peptide, a protein, a nucleotide, an oligonucleotide, a nucleic acid polymer, a carbohydrate, a lipid, an ion-complexing moiety, or a non-biological polymer.

5. A compound as claimed in Claim 1, wherein Sc is an amino acid, a peptide, a protein, a nucleotide, an oligonucleotide, a nucleic acid polymer, a carbohydrate, a lipid, or a drug.
6. A compound, as claimed in Claim 1, wherein Sc is a hapten or a member of a specific binding pair.
- 5 7. A compound as claimed in Claim 1, wherein Sc is further conjugated to one or more luminophores, where each luminophore may be the same or different.
8. A compound, as claimed in Claim 7, wherein at least one of the luminophores is a fluorophore.
- 10 9. A compound, as claimed in Claim 8, wherein the fluorophore is a pyrene, an anthracene, a naphthalene, an acridine, a stilbene, an indole, a benzindole, an oxazole, a benzoxazole, a thiazole, a benzothiazole, a 4-amino-7-nitrobenz-2-oxa-1,3-diazole, a cyanine, a carbocyanine, a carbostyryl, a porphyrin, a salicylate, an anthranilate, an azulene, a perylene, a pyridine, a quinoline, a coumarin, 15 a polyazaindacene, a xanthene, an oxazine, a benzoxazine, a carbazine, a phenalenone, or a benzphenalenone.
10. A compound, as claimed in Claim 8, wherein the fluorophore is a carbazine, an oxazine, a coumarin, a pyrene, a xanthene, a naphthalene, a phenalenone, or a 4-bora-3a,4a-diaza-s-indacene.
- 20 11. A compound, as claimed in Claim 8, wherein the fluorophore is a fluorescein, a rhodamine, or a rhodol.
12. A compound as claimed in Claim 1, wherein each Q moiety is a substituted or unsubstituted 25 phenyl, naphthyl, anthracenyl, benzothiazole, benzoxazole, or benzimidazole.
13. A compound, as claimed in Claim 1, wherein each Q moiety is a phenyl or substituted phenyl.
14. A compound, as claimed in Claim 1, wherein K is  $N \cdot R^{18}R^{19}$ .
- 30 15. A compound, as claimed in Claim 1, wherein  $R^8$  taken in combination with  $R^9$  forms a saturated 5- or 6-membered heterocycle that is fused to a Q moiety, and  $R^{18}$  taken in combination with  $R^{19}$  forms a saturated 5- or 6-membered heterocycle that is fused to a Q moiety.
- 35 16. A compound, as claimed in Claim 1, wherein  $R^8 = R^{19}$  and  $R^9 = R^{18}$ .
17. A compound, as claimed in Claim 1, wherein  $R^1$ ,  $R^2$ ,  $R^5$ , and  $R^6$  are each H.

18. A compound, as claimed in Claim 1, wherein  $R^{10}$  has the formula

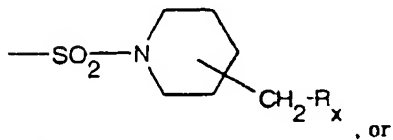
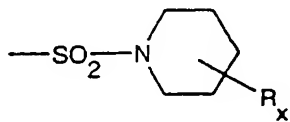


19. A compound, as claimed in Claim 18, wherein one of  $R^{12}$ - $R^{16}$  is  $-L-R_x$  or  $-L-Sc$ .

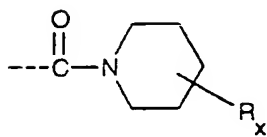
20. A compound, as claimed in Claim 18, wherein  $R^{12}$  is  $-L-R_x$ ;

wherein L is selected such that  $-L-R_x$  has the formula

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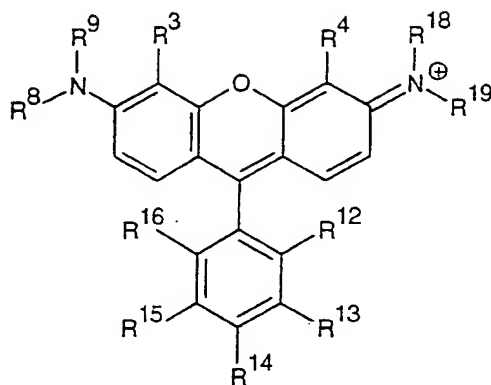


, or



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21. A compound, as claimed in Claim 1, having the formula



wherein

5  $R^8 = R^{19}$  and  $R^9 = R^{18}$ ;

$R^{12}$  is  $-L-R_x$ , or  $-L-Sc$ ; and

each Q moiety is a substituted or unsubstituted phenyl, naphthyl, anthracenyl, benzothiazole,  
10 benzoxazole, or benzimidazole.

22. A compound, as claimed in Claim 1, having a fluorescence quantum yield of less than about 0.05.

23. A method of detecting a change in separation distance between one or more luminophore donors  
15 and quenching compound acceptors in a sample, wherein at least one quenching compound is defined as in one of Claims 1-22, said method comprising the steps of:

- a) illuminating said sample;
- 20 b) detecting a first luminescence response of said sample;
- c) exposing said sample to an environmental condition expected to change said separation distance;
- d) illuminating said sample;
- 25 e) detecting a second luminescence response of said sample; and

f) comparing said first and second luminescence response to determine a detectable difference in luminescence, where said detectable difference in luminescence correlates with said change in separation distance.

5 24. A method, as claimed in Claim 23, wherein said detectable difference is an increase in luminescence.

25. A method, as claimed in Claim 23, wherein said detectable difference is a decrease in luminescence.

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26. A method, as claimed in Claim 23, wherein said luminescence is fluorescence.

27. A method, as claimed in Claim 23, wherein Sc is an oligonucleotide, nucleic acid polymer, peptide or protein.

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28. A method, as claimed in Claim 23, wherein Sc is a member of a specific binding pair.

29. A method, as claimed in Claim 23, wherein said quenching compound acceptor and said luminophore donor are initially covalently conjugated to the same conjugated substance Sc.

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30. A method, as claimed in Claim 29, wherein the conjugated substance is cleaved by said environmental condition, resulting in an increase in luminescence.

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31. A method, as claimed in claim 29, wherein the conjugated substance changes conformation in response to said environmental condition, resulting in said detectable difference in luminescence.

32. A kit for labeling a substance, comprising:

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a) a quenching compound as claimed in one of Claims 1-22 wherein at least one of R<sup>8</sup>, R<sup>9</sup>, R<sup>12</sup>, R<sup>13</sup>, R<sup>14</sup>, R<sup>15</sup>, R<sup>16</sup>, R<sup>18</sup>, or R<sup>19</sup> is -L-R<sub>x</sub>; or at least one Q moiety is substituted by -L-R<sub>x</sub>;

b) instructions for conjugating said quenching compound to said substance; and

c) at least one of the following:

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i) a buffering agent;

ii) a purification medium;

iii) a sample of said substance;

iv) an organic solvent;

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v) an enzyme; or

vi) an enzyme inhibitor.

10 33. A kit, as claimed in Claim 32, wherein said substance is an oligonucleotide, nucleic acid polymer, peptide or protein.

34. A kit, as claimed in Claim 32, wherein said substance is a member of a specific binding pair.

15 35. A kit, comprising:

a) a quenching compound as claimed in one of Claims 1-22 wherein at least one of R<sup>8</sup>, R<sup>9</sup>, R<sup>12</sup>, R<sup>13</sup>, R<sup>14</sup>, R<sup>15</sup>, R<sup>16</sup>, or R<sup>19</sup> is -L-Sc; or at least one Q moiety is substituted by -L-Sc; and

20 b) a luminophore donor.

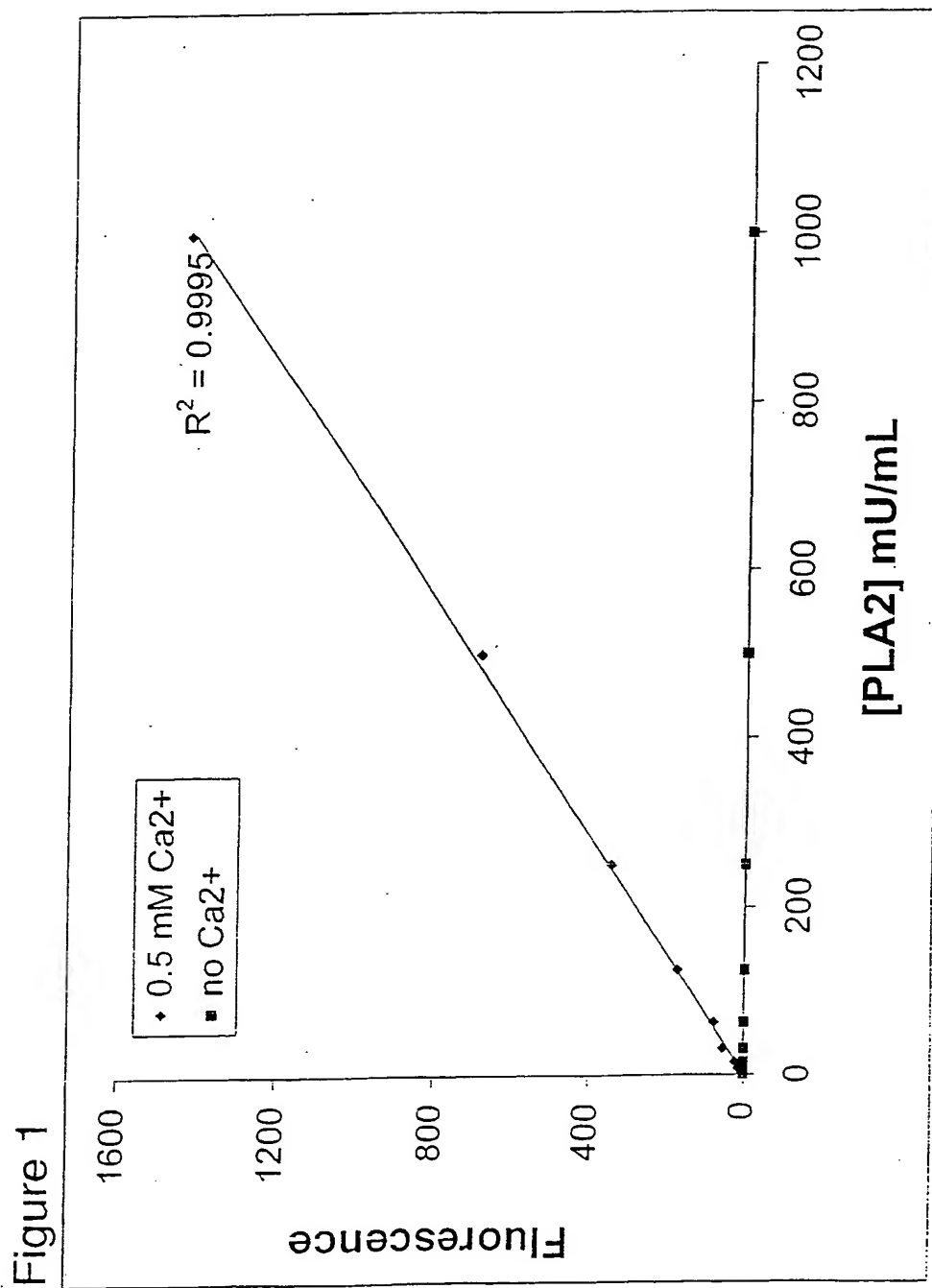
36. A kit, as claimed in Claim 35, further comprising an additional detection reagent.

37. A kit, as claimed in Claim 35, further comprising one or more luminescence standards.

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38. A kit, as claimed in Claim 35, wherein said said luminophore donor is also covalently bound to the conjugated substance Sc.

39. A kit, as claimed in Claim 35, wherein Sc is a member of a specific binding pair, and said  
30 luminophore donor is covalently bound to the complementary member of said specific binding pair.



# INTERNATIONAL SEARCH REPORT

International Application No <b>PCT/US 00/10740</b>		
<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC 7 C09B11/24 G01N33/533 G01N33/58 G01N33/542 C12Q1/68 C07H21/00 //C09B11/02, C12Q1/34, C12Q1/37		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) IPC 7 C09B G01N C12Q C07H		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) <b>Biosis, Chem Abs Data, EPO-Internal</b>		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 99 16832 A (PERKIN ELMER CORP) 8 April 1999 (1999-04-08) page 2, line 10 -page 5, line 18; claims ---	1-39
A	WO 99 15517 A (LEUNG WAI YEE ;MAO FEI (US); HAUGLAND RICHARD P (US); MOLECULAR PR) 1 April 1999 (1999-04-01) cited in the application claims; examples ---	1-39
A	EP 0 805 190 A (PERKIN ELMER CORP) 5 November 1997 (1997-11-05) claims; examples; tables 1,2,4-6 ---	1-39
A	WO 97 39064 A (MOLECULAR PROBES INC) 23 October 1997 (1997-10-23) cited in the application claims 3-5,15,18-26 ---	1-39
-/--		
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex		
* Special categories of cited documents : <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Z" document member of the same patent family</p> </div> </div>		
Date of the actual completion of the international search  <b>11 August 2000</b>	Date of mailing of the international search report  <b>21/08/2000</b>	
Name and mailing address of the ISA European Patent Office, P.O. 5816 Patentaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 600 nl, Fax: (+31-70) 340-3016	Authorized officer  <b>Ginoux, C</b>	



# INTERNATIONAL SEARCH REPORT

Inter. nat Application No

PCT/US 00/10740

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 227 487 A (HAUGLAND RICHARD P ET AL) 13 July 1993 (1993-07-13) cited in the application claims	1-39

# INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/US 00/10740

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